REMARKS/ARGUMENTS

Claims 26-44 are pending. While Applicants believe that none of the cited references shows that leflunomide products are efficacious for treating viral infection, Applicants have amended claim 26 to exclude hepatitis and AIDS solely to expedite prosecution. Support for this amendment in the form of reference to hepatitis viruses and HIV as separate categories of viruses is found throughout the application, e.g. at page 16, lines 14-15 and page 18, lines 10-11.

Independent claim 34, as amended, addresses combination therapy of viral infection using leflunomide product and a pyrimidine. Support for the amendment to claim 34 is found, e.g., at page 16, lines 5-7 and page 20, lines 1-6. Support for the exemplary pyrimidines listed in new claims 41-42 is found, e.g., at 20, lines 14-15.

Support for the amides of malononitriles as described in claims 37 and 43 is found at page 19, line 2. Exemplary malononitriles are shown at page 19 and in U.S. Patent Nos. 4,087,535 and 5,519,042, which were specifically incorporated by reference into the present specification at page 18, lines 16-17. The specification has been amended to insert the text from col. 2, line 33 through col. 3, line 9 of U.S. Patent No. 5,519,042 (describing leflunomide products of formulas I and II) that was incorporated by reference. Support for the formula recited in claims 38 and 44 is found in formula II which was added to the specification by this amendment.

Applicants thank Examiner Wang for the courtesy of the telephonic interview kindly granted to the undersigned on February 21, 2006, during which the amendment to claim 26 and the nonobviousness of the combination therapy of claim 34 was discussed. Although agreement was not reached as to patentability, the Examiner agreed to consider Applicants' amendments and arguments.

The rejection under 35 U.S.C. 102(b) over Weithmann is moot in view of the amendment to claim 26. Regarding the rejection under 35 U.S.C. 103 over Weithmann and Flamand et al., Applicants respectfully submit that the combination of these two references is not proper and moreover does not render the claims obvious for the following reasons: (1) one of ordinary skill in the art is *not motivated to administer an immunosuppressant such as leflunomide to treat infection* because a non-suppressed immune system is desirable to combat infection, (2) Flamand et al. does not show that IL-1 beta levels are actually elevated or (even if elevated) deleterious *in animals* infected by herpesvirus, (3) the cited art provides *no reasonable expectation of success*

that leflunomide would in fact have a beneficial effect on IL-1 beta levels in animals infected by herpesvirus, (4) neither reference teaches the *unexpected effects on viral virion assembly* demonstrated by Applicants, and (5) neither reference teaches one to administer an *amount effective* to inhibit viral growth. Although the Examiner has taken the position that optimization of dosages is routine, it is not obvious to optimize a dosage to achieve an effect (inhibition of viral growth rather than effect on IL-1 beta levels) that is not known or suggested in the cited art.

In contrast, Applicants have shown in the specification that leflunomide products inhibit viral growth of a number of different viruses *in vitro*. Applicants have confirmed *in vivo* activity in Example 7, which shows that administration of leflunomide to rats infected with CMV reduces their viral load. See also Waldman et al., Intervirology, 1999;42(5-6):412-8, "Inhibition of cytomegalovirus in vitro and in vivo by the experimental immunosuppressive agent leflunomide" [Exhibit A hereto].

For these reasons, claim 26 and all claims 27-33 and 43-44 dependent thereon are believed to be patentable.

Applicants respectfully request that the Examiner consider patentability of the combination therapy claims 34-42 on a separate basis in view of the unexpected effects of the combination disclosed in the instant application, and in view of the claim's exclusion of antiviral nucleoside analogs that do not enhance serum levels of uridine, cytidine or thymidine. Claim 34 was rejected under 35 U.S.C. 103(a) over Coghlan et al. in view of McChesney et al. and Hammer. It was the Examiner's position that Hammer teaches that pyrimidine compounds are known anti-viral agents, and that it would be obvious to combine two anti-viral agents. As noted above, Applicants believe the Examiner is mistaken in his belief that the art teaches the anti-viral effect of leflunomide products. However, even if one assumes for the sake of argument that the art does contain such a teaching, Applicants respectfully submit that the Examiner is mistaken in his interpretation of what is disclosed in Hammer.

Hammer teaches the anti-HIV effects of nucleoside *analogs*, which *do not* enhance serum levels of pyrimidines (uridine, cytidine or thymidine) as recited in claim 34 and thus are a *different category of compounds*. As noted in the specification at page 20, lines 12-14, compounds that enhance serum levels of pyrimidines are compounds useful either directly or as intermediates in pathways for supplying pyrimidines. In contrast, anti-retroviral nucleoside analogs act by binding to reverse transcriptase, thereby inhibiting viral DNA synthesis by chain

termination. Thus, the basis for their anti-retroviral activity is their ability to act *unlike natural nucleosides*, and this anti-retroviral activity is accompanied by toxic effects that are reversible by administration of selected natural pyrimidines. See Sommadossi et al., *Antimicrob. Agents Chemother.* 32(7): 997-1001 (1988) and Walker et al., *Antivir. Ther.* 10 suppl. 2:M117-23 (2005) (abstract) [Exhibits B and C hereto, respectively], which disclose that uridine can reduce the toxic effects of administering nucleoside analog reverse transcriptase inhibitors.

Thus, Hammer's disclosure with respect to nucleoside analogs teaches nothing with respect to the pyrimidine compounds recited in claim 34. Moreover, Hammer contains absolutely no suggestion that uridine, orotic acid or orotidine recited in claims 41-42 have anti-viral activity. For these reasons, it would not be obvious to administer a pyrimidine that enhances serum levels of uridine, cytidine or thymidine to a person suffering from viral infection according to claim 34.

Moreover, such combination therapy is also unobvious because of the unexpected results of the combination, *i.e.* the reduction in the toxic side effects of leflunomide product. See page 20, lines 1-6. Evidence confirming these unexpected results is found, e.g., in Chong et al., Transplantation, 1999 Jul 15;68(1):100-9 [Exhibit D hereto], which states in the abstract that:

> Toxicities associated with high-dose leflunomide (35 mg/kg/day) were anemia, diarrhea, and pathological changes in the small bowel and liver. *These toxicities were significantly reduced by uridine co-administration.* [Emphasis added.]

WO 2006/014827 [Exhibit E hereto] shows that another pyrimidine, orotic acid, also reduces the toxic side effects of leflunomide. The present application shows that the reduction in toxic side effects produced by pyrimidine administration does not interfere with the anti-viral activity of leflunomide product. Example 2 of the specification at pages 26-27 indicates that co-treatment of virally infected cells with A771726 and uridine did not interfere with the anti-viral activity of A771726 because infectious virus production was not significantly reconstituted in the presence of uridine.

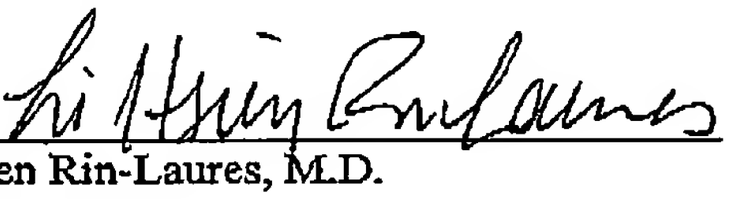
All claims 35-42 which depend from claim 34 are believed to be patentable for similar reasons. If the Examiner believes that a telephonic interview would expedite prosecution, the Examiner is encouraged to contact the undersigned.

No additional fees are believed to be necessary in connection with the present Amendment. However, the Commissioner is hereby authorized to charge any fees due or deficiency in the fees submitted to our Deposit Account No. 13-2855, under Order No. 28385/35415.

Respectfully submitted,

MARSHALL, GERSTEIN & BORUN LLP

February 27, 2006



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Part II. Diagnostics and Antiviral Therapy
Antivirals

Exhibit A

Intervirology

Intervirology 1999;42:412–418

Inhibition of Cytomegalovirus in vitro and in vivo by the Experimental Immunosuppressive Agent Leflunomide

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Key Words
Cytomegalovirus · Leflunomide · Antiviral therapy · Endothelial cells · Fibroblasts

Abstract
Despite progress in antiviral chemotherapy, cytomegalovirus (CMV) remains a major cause of morbidity and mortality among pharmacologically immunosuppressed transplant recipients, frequently engaging the clinician in a struggle to balance graft preservation with control of CMV disease. Leflunomide, an inhibitor of protein kinase activity and pyrimidine synthesis, is an experimental immunosuppressive agent effective against acute and chronic rejection in animal models. Herein we summarize our recent studies demonstrating that leflunomide inhibits the production of multiple clinical CMV isolates (including multi-drug-resistant virus) in both human fibroblasts and endothelial cells. In contrast to all other anti-CMV drugs currently in use, leflunomide does not inhibit viral DNA synthesis, but rather appears to interfere with virion assembly. Finally, preliminary studies in a rat model suggest that this agent reduces viral load in vivo. These findings imply that leflunomide, an effective immunosuppressive agent, shows potential to concurrently attenuate a major complication of immunosuppression, CMV disease, by a novel mechanism of antiviral activity.

Introduction

Despite widespread prevalence, cytomegalovirus (CMV) infection is rarely of significant consequence in the healthy individual with a competent immune system. However, the progression of the AIDS epidemic and experience with the rapidly growing population of transplant recipients emphasize that this β-herpesvirus can be clinically problematic among immunosuppressed populations. HIV-induced erosion of cellular immunity promotes reactivation of endogenous latent CMV in AIDS patients and renders them highly susceptible to symptomatic primary infection. Pharmacologic immunosuppression required for preservation of allograft integrity similarly disables host defenses in transplant recipients. Thus, despite refinements in therapeutic intervention in these patients, CMV remains a source of a diverse constellation of serious, often life-threatening complications, including interstitial pneumonitis, diffuse gastrointestinal mucosal

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ulceration, hepatitis, and retinitis, as well as destructive inflammatory lesions in a variety of other locations [1]. In addition, several studies have suggested a role for this virus as a contributing factor in allograft rejection [2, 3].

Currently, three compounds, ganciclovir (GVC), foscarnet (PFA) and cidofovir, are approved for clinical use in the control of CMV disease, none of which are free of toxic side effects. Drug-associated myelosuppression, metabolic toxicity, and nephrotoxicity limit the use of these agents in a significant number of patients [4–6]. Although specific mechanisms of action vary among these compounds, all three ultimately act by inhibition of viral DNA synthesis, either by impeding chain elongation following incorporation into nascent strands [4, 7], or by direct binding to viral DNA polymerase [6]. It is not surprising, therefore, that clinical strains of CMV have emerged which exhibit resistance to one or more of these drugs [8–10]. Clearly there remains a need for development of additional therapeutic approaches.

Leflunomide [N-(4-trifluoromethylphenyl)-methylisoxazol-4-carboxamide; HWA 486] is an experimental immunosuppressive agent with demonstrated effectiveness in the prevention and reversal of acute allograft rejection in rats and dogs [11, 12], as well as in the reversal of chronic rejection when combined with cyclosporine [13]. It has also been shown to be effective against rat and murine graft-versus-host disease and against autoimmune disease in animal models [14]. Leflunomide has recently been approved for treatment of rheumatoid arthritis, and has been well tolerated in current phase I clinical trials in human transplant recipients [Dr. James W. Williams, Rush presbyterian St. Luke's Medical Center, Chicago, Ill. pers. commun.]. This isoxazol derivative is metabolized to its active form, A77 1726 [N-(4-trifluoromethylphenyl)-2-cyano-3-hydroxycrotoamide], which is structurally unique among established immunosuppressive agents [14]. This metabolite exhibits two known mechanisms of action: inhibition of protein tyrosine kinase activity [15], and inhibition of dihydroorotate dehydrogenase, a key enzyme in the biosynthesis of pyrimidine nucleotide triphosphates (pyNTP) [16]. However, neither the complete spectrum of kinases targeted by leflunomide nor the specific contribution of each of these functions to its immunosuppressive activity in vivo has been fully resolved. Furthermore, additional mechanisms of activity may yet remain to be discovered.

Based upon the fact that a number of CMV-encoded proteins are phosphoproteins, and that several CMV proteins themselves possess kinase activity [17], we speculated that protein kinase activity may be essential in intracellular production of infectious virus. Thus we tested the hypothesis that leflunomide might exert inhibitory activity against CMV in human fibroblasts and endothelial cells, common targets of CMV infection in vivo [18, 19]. As summarized below, we have shown that this is indeed the case, and have further demonstrated the mechanism of viral inhibition by leflunomide to be unique among currently approved anti-CMV therapeutic agents. Finally, preliminary experiments in a rat model suggest that this agent is capable of reducing viral load in vivo.

Methods and Results

Effect of Leflunomide upon the Production of Infectious CMV in vitro

Our first evidene for an antiviral effect of leflunomide came from microscopic observation of human umbilical vein endothelial cell (HUVEC) monolayers inoculated at low titer with CMV VHL/E, a clinical isolate propagated in HUVEC to preserve its natural endothelial cytopathogenicity [20], and incubated in the presence or absence of A77 1726 (200 μM), the active metabolite of leflunomide [14]. This isolate induces obvious cytopathic change and, under normal circumstances, disseminates by direct cell-to-cell transmission generating widespread cytopathology over a period of several days. In the presence of A77 1726, however, this restriction was dramatically restricted [21]. Similar patterns of dissemination and A77 1726-mediated restriction were evident in human fibroblast (HFF) monolayers inoculated with clinical isolate P8 (isolated from bronchial alveolar lavage obtained from a cardiac allograft recipient and propagated in HFF cells [10]).

To quantitatively assess the impact of leflunomide upon the production of infectious virus in HUVEC and HFF, we performed standard plaque reduction and virus yield assays in the presence of A77 1726 over a range of concentrations equivalent to those which have been shown to attenuate immune activation by various stimuli in vitro [22], and equivalent to (or less than) well-tolerated serum levels measured in leflunomide-treated experimental animals [23] and human clinical trial subjects (270–450 μM) [Dr. James W. Williams, unpubl. data]. Data generated by these experiments demonstrated an A77 1726-mediated, dose-dependent reduction in infectious virus production in both cell types. The dose-response curves of clinical isolate P8 in HFF and of clinical isolate VHL/E in HUVEC did not differ significantly, both indicating an IC_{50} of 40–60 μM [21]. Similar assays performed with CMV VHL/E and an additional clinical

isolate, BUR/E [24], in the presence of cyclosporine A (CsA) or tacrolimus (FK 506), verified that neither of these commonly prescribed immunosuppressive agents possesses antiviral activity against CMV [21].

Effect of Exogenous Uridine upon the Antiviral Activity of Leflunomide

Two mechanisms have been identified for the activity of A77 1726: inhibition of protein tyrosine kinase activity [15], and inhibition of dihydroorotate dehydrogenase [16], a key enzyme in the biosynthesis of pyNTP. To determine whether reduction of intracellular pyNTP pools was responsible for the antiviral effects of leflunomide, HUVEC were inoculated with CMV VHL/E, then incubated for 4 days in the presence or absence of 200 μ*M* A77 1726, 200 μ*M* exogenous uridine, or both. Uninfected HUVEC were included in each experiment as negative controls. Following harvest, cells were extracted by methods described by Khym [25] and intracellular NTP levels were analyzed by high-performance liquid chromatography. Small aliquots of cells from each group were reserved prior to extraction and assayed for virus yield by plaque assay.

Data generated by these experiments demonstrated several trends, none of which reached statistical significance as determined by ANOVA. First, intracellular pyNTP levels were slightly reduced in untreated CMV-infected HUVEC as compared to uninfected control cells, but were not further reduced by A77 1726 treatment. Second, the addition of exogenous uridine increased pyNTP levels both in the presence and absence of A77 1726. Most importantly, however, the addition of exogenous uridine did not significantly reconstitute infectious virus production in A77 1726-treated, CMV-infected HUVEC [21]. Thus A77 1726-mediated inhibition of CMV activity appears to be independent of the inhibitory effects of this agent upon pyNTP synthesis.

Effects of Leflunomide upon CMV Gene Transcription and Protein Expression

We next sought to determine at which point in the viral replication cycle leflunomide exerts its inhibitory effects. Immediately upon viral entry of the host cell, CMV lower matrix protein pp65, a component of the viral tegument, translocates to the cell nucleus. Although the nuclear function of pp65 remains to be resolved, another tegument protein, pp71, which migrates in a similar manner, acts in concert with cellular proteins to promote rapid transcription of immediate early (IE) viral genes. IE gene products are primarily regulatory, activating early gene transcription, whose products are essential for viral DNA replication. Activation of late genes, which code primarily for structural protein components of the virion, occurs late in the replicative cycle and is dependent upon viral DNA replication [17].

To localize potential A77 1726-induced lesions in this temporal series of events, we first visualized a cross-section of CMV proteins in A77 1726-treated CMV-infected HUVEC by immunohistochemical staining with monoclonal antibody (mAb) specific for pp65, 72 kD IE1, or late structural glycoprotein B (gB). Staining patterns indicated that none of these viral proteins were prevented from being expressed in A77 1726-treated cells. Nuclear accumulation of pp65 was apparent in both untreated and A77 1726-treated monolayers within hours after inoculation, implying that leflunomide interferes neither with viral entry, nor with nuclear translocation of pp65. Nuclear IE1 expression also appeared to be unaffected by A77 1726. Finally, typical gB staining patterns were apparent at 72–96 h after inoculation in both treated and untreated cultures. In contrast, infected cells treated with PFA, an inhibitor of CMV DNA polymerase, were unable to express gB, while pp65 and IE1 expression in these cultures was unimpeded [21].

We next employed Northern blot analysis to determine whether A77 1726 quantitatively affected IE1 or gB transcription. Total cytoplasmic RNA was isolated from CMV-inoculated HUVEC incubated for 48 h in the absence or presence of 200 μ*M* A77 1726, 1 m*M* PFA, or 1.2 m*M* ganciclovir (GCV). Each experiment also included RNA isolated from uninfected HUVEC (as negative control). Electrophoretically fractionated RNA was transferred to Nylon membranes and hybridized to [^{32}P]-labeled probes specific for CMV IE1, CMV gB, or cellular GAPDH (as a loading control), and bands were visualized by autoradiography. The results demonstrated that, in contrast to PFA and GCV which completely suppressed gB transcription, A77 1726 had no apparent effect upon the transcription of either IE1 or gB [21].

Effect of Leflunomide upon CMV DNA Synthesis

Since expression of late structural proteins (such as gB) is dependent upon CMV DNA replication [17], our immunohistochemical and Northern blot analyses suggested that, in contrast to currently used anti-CMV therapeutics, leflunomide does not inhibit viral DNA synthesis. The potential novelty of such an alternative antiviral mechanism led us to employ three independent experimental approaches to test this hypothesis. First we measured the incorporation of [^{3}H]thymidine in HUVEC or HFF

which had been 100% infected with CMV VHL/E or P8, respectively, and incubated for 36 h in the presence or absence of various concentrations of A77 1726. While PFA inhibited radiolabel incorporation by CMV-infected cells in a dose-dependent manner, no inhibition was detected in the presence of A77 1726 [21].

We next generated dot blots of serially diluted DNA extracted from P8-infected HFF or VHL/E-infected HUVEC which had been incubated for 48 h after inoculation in the presence or absence of 100 μ*M* (HFF) or 200 μ*M* (HUVEC) A77 1726, or 1 m*M* PFA. Blots were hybridized to [^{32}P]-labeled CMV-specific cDNA probe and visualized by autoradiography. These studies demonstrated that quantities of viral DNA synthesized in CMV-infected cells incubated in the presence of A77 1726 were approximately equivalent to those accumulated in untreated infected cells. In contrast, PFA-treated cells contained no detectable CMV DNA. The specificity of the probe was verified by the absence of hybridization to DNA extracted from uninfected cells [21].

Finally to directly test the effect of leflunomide upon viral DNA polymerase, crude protein extracts were prepared from CMV-infected (or uninfected) HFF or HUVEC, and viral DNA polymerase activity was measured in the presence of various concentrations of A77 1726 by biochemical assay of enzyme-catalyzed incorporation of [^{3}H]TTP into nicked template DNA. While PFA reduced viral DNA polymerase activity in a concentration-dependent manner, A77 1726 showed no detectable inhibitory activity even at concentrations which dramatically reduced plaque formation. Experiments performed with extracts prepared from CMV VHL/E or BUR/E-infected HUVEC, or P8-infected fibroblasts generated essentially identical results. No enzyme activity was detected in extracts prepared from uninfected cells [21].

Effect of Leflunomide upon Virion Morphology

Collectively, results of our immunohistochemical staining, Northern blot analysis, viral DNA blots, and polymerase assays argue in favor of a leflunomide-associated antiviral mechanism which is unique with respect to other anti-herpesvirus compounds. Since three of the five major CMV tegument proteins are phosphoproteins [26], we hypothesized that leflunomide-mediated inhibition of protein phosphorylation might disable processes essential in the maturation and assembly of the complete viral particle. To address this issue, we employed transmission electron microscopy to directly examine virion morphology within A77 1726-treated or untreated CMV-infected cells at 4–7 days after inoculation. Electron micrographs demonstrated typical herpesvirus capsids within the nuclei of A77 1726-treated cells, implying that neither nucleocapsid assembly nor viral DNA packaging is affected by this agent. However, we observed profound differences in the morphology of virions maturing in the cytoplasm. While tegument and external membrane were acquired normally in untreated cells, viral particles appeared not to mature beyond the 100 nm naked capsid stage in the presence of A77 1726 [21].

Activity of Leflunomide against Multi-Drug-Resistant CMV

All currently approved anti-CMV chemotherapies focus upon inhibition of viral DNA synthesis, although specific mechanisms vary among different agents [4, 6, 7] (see Discussion). Thus it is not surprising that multi-resistant clinical strains have emerged [8–10]. Based upon our discovery of the apparently unique mechanism of viral inhibition by leflunomide, we tested the hypothesis that this agent might suppress activity of drug-resistant virus. CMV strain D16 was isolated from the same patient as strain P8. However, unlike P8, D16 exhibits resistance to GCV, PFA, and cidofovir [10]. Plaque reduction assays performed in HFF cultures revealed equivalent sensitivity of these two isolates to A77 1726 (IC_{50} ~ 40–60 μ*M*) [21]. Thus leflunomide-mediated inhibition of production of D16 is independent of resistance to current clinically applied chemotherapeutic agents.

Antiviral Activity of Leflunomide in vivo

To determine the effectiveness of leflunomide in the control of viral load in vivo, groups of immunodeficient nude rats were inoculated with rat CMV (RCMV Maastricht strain [27]. 10^5 plaque-forming units/animal) and treated with either leflunomide (15 mg/kg/day for 14 days), GCV (10 mg/kg/day for 5 days), or drug-free vehicle. Experiments also included uninfected control animals. Following euthanization at 14 days after inoculation, viral infection was confirmed by histologic observation of typical cytomegalic ductal epithelium in salivary glands, and further verified by immunohistochemical detection of RCMV antigens in multiple tissues. Plaque assay of tissue homogenates prepared from salivary gland, spleen, and lung demonstrated 75–99% reduction in virus yield in organs harvested from leflunomide-treated animals, and 85–99% reduction in those harvested from ganciclovir-treated animals. No virus was ever recovered from uninfected control rats [28]. Thus in addition to its in vitro antiviral activity, leflunomide is capable of reducing viral load in vivo.

Discussion

Despite substantial progress in antiviral chemotherapy and prophylaxis, CMV infection remains a significant complicating factor in the clinical course of up to 50% of organ transplant recipients [1]. Thus, fine tuning of pharmacologic immunosuppression to maintain a balance between graft preservation and control of CMV disease presents a persistent problem for the clinician. Results of the studies summarized herein suggest that leflunomide may help to alleviate this dilemma. Specifically, we have demonstrated that A77 1726, the active metabolite of leflunomide, inhibits infectious CMV production in a dose-dependent manner over a range of concentrations similar to those which inhibit in vitro T cell activation by mitogenic or allogeneic stimuli [22], and equivalent to (or less than) well-tolerated serum levels measured in leflunomide-treated experimental animals [23] and human clinical trial subjects [Dr. James W. Williams, unpubl. data]. Similar patterns of viral inhibition were observed for each of 4 individual clinical CMV isolates, and in both human fibroblasts and human endothelial cells, common targets of CMV infection in vivo [18, 19]. Furthermore no such inhbitory activity was observed for either CsA or FK 506 [21].

Which of the two known mechanisms of leflunomide, inhibition of pyrimidine synthesis or inhibition of protein tyrosine kinase activity, contributes most significantly to its immunosuppressive properties remains to be resolved. However, our experiments clearly show that the antiviral activity of this agent cannot be attributed to a reduction in intracellular pyNTP pools [21]. While previous studies have demonstrated profound pyNTP depletion in IL-2-stimulated murine T cells [29] and murine leukemia cells [30] cultured in the presence of A77 1726, we observed little if any A77 1726-associated CTP or UTP reduction in CMV-infected endothelial cells. Although these outcomes might seem at first contradictory, it should be noted that in those previous studies the murine cells were vigorously proliferating prior to leflunomide treatment. In contrast, HUVEC assayed in our experiments were in a stationary, quiescent phase. Thus, with the exception of viral DNA replication, minimal intracellular NTP consumption would be expected. We note that, although addition of exogenous uridine increased intracellular pyNTP concentrations both in the presence and absence of A77 1726, it did not significantly reconstitute infectious virus production in A77 1726-treated, CMV-infected cells, nor did it correct A77 1726-associated defects in viral particle asssembly [21]. These data argue against the hypothesis that pyNTP depletion is responsible for the antiviral activity of leflunomide.

The results of our experiments directed toward identification of specific viral processes targeted by leflunomide suggest mechanisms that are unique among anti-CMV therapeutic agents in use at present. GCV, currently the drug of choice for the treatment of CMV disease, is a guanosine analogue which is monophosphorylated in infected cells by the protein product of the CMV UL97 gene [31, 32]. Cellular kinases convert the monophosphate form to a triphosphate which is then incorporated by CMV DNA polymerase into the replicating viral DNA where its presence inhibits chain elongation [4]. PFA has been used increasingly as an alternative to GCV, in particular when GCV resistance is suspected [6]. This agent also inhibits CMV DNA replication but does so by binding directly to the viral DNA polymerase [6]. Cidofovir, the newest addition to the anti-CMV armamentarium, recently approved for use in the control of CMV retinitis [33], is a monophosphorylated cytosine analogue which, like GCV, is further phosphorylated by cellular kinases, ultimately inhibiting viral DNA synthesis following incorporation into nascent strands [7].

In contrast to these agents (as well as several others with similar activities against other herpesviruses), our studies indicate that leflunomide does not inhibit CMV DNA replication [21]. This was initially suggested by immunohistochemical staining and Northern blot analysis demonstrating that A77 1726 had no effect upon expression of CMV late structural protein gB or transcription of its mRNA, both of which are dependent upon viral DNA synthesis. Subsequent measurement of [^{3}H]thymidine incorporation by infected cells, viral DNA dot blot analysis, and biochemical assay of viral DNA polymerase activity, provided proof that indeed this agent exerts no inhibitory effect upon the accumulation of viral DNA or upon the activity of the enzyme. Rather, the major A77 1726-mediated lesion in infectious virus production appears to be in the process of maturation and assembly of the complete virion, specifically (as demonstrated by transmission electron microscopy) failure to acquire tegument and external membrane in the cytoplasm of infected cells [21]. Although little is presently known of the structural organization of the CMV tegument, three of the five major tegument proteins are phosphoproteins (pp150, pp71, pp65) [26]. Thus we speculate that by inhibiting protein phosphorylation, leflunomide may interfere with tegument assembly. Experiments are currently in progress to test this hypothesis and to identify specific proteins whose phosphorylation is inhibited by A77 1726. Out-

comes of these studies may implicate this compound as a useful reagent in further investigations of mechanisms of virion maturation.

The emergence of drug-resistant variants of CMV in response to antiviral agents increasingly complicates treatment. Resistance to GCV is primarily due to mutation of the CMV UL97 gene which codes for the phosphotransferase responsible for the initial monophosphorylation of this compound [31, 32]. Several different mutations have been reported, all of which result in single amino acid substitutions or short deletions in the UL97 protein product [8]. In addition in the viral DNA polymerase gene (UL54) resulting in single amino acid substitutions have also been detected in GCV-resistant isolates [8]. Unlike GCV, PFA requires no anabolic processing to enable its binding to viral DNA polymerase [6]. Thus resistance to PFA appears to result exclusively from UL54 mutations [6, 8]. Similarly, the molecular basis for cidofovir resistance has thus far been traced exclusively to UL54 mutations [9]. While the mechanisms of action of these agents vary in detail, all three ultimately target viral DNA polymerization as a common endpoint. Moreover, it is not surprising that CMV variants which develop resistance to one of these drugs can often exhibit resistance to others [8–10]. It is also not surprising that leflunomide, as a consequence of its distinctly different mechanism of action, can exert inhibitory activity against drug-resistant CMV equal to that against drug-sensitive isolates.

Leflunomide, recently approved for the treatment of rheumatoid arthritis, has been shown to be effective in the prevention and reversal of acute and chronic allograft rejection in several animal transplantation models [11–13]. The reproducible success of those animal experiments and the documented low toxicity of this agent [34] have led to its current status as a candidate immunosuppressant in phase I clinical trials in human transplant recipients. Findings generated in the current investigation indicate that leflunomide also possesses an additional unexpected beneficial property that distinguishes it from other immunosuppressive drugs: antiviral activity against CMV. Furthermore this agent, likely by virtue of its unique mechanism of action, shows equivalent effectiveness against CMV variants which have developed resistance to approved antiviral drugs.

Remaining to be further substantiated, however, is whether leflunomide can exert antiviral effects in vivo. Although additional studies are needed, our preliminary experiments in a rat model indicate it can [28]. Should subsequent large-scale animal studies support these preliminary results, this agent would show great potential in the clinical setting. Although it seems overly optimistic to expect leflunomide to provide a single drug solution for both allograft rejection and CMV disease in the transplant recipient, an effective immunosuppressant that substantially reduces viral load would greatly simplify treatment protocols employing traditional antiviral agents. Specifically, additive or synergistic effects between leflunomide and GCV, PFA, or cidofovir could be expected to reduce the required dosage and/or duration of treatment, thereby reducing untoward side-effects. In summary, by virtue of its low toxicity [34], its apparent bifunctionality as both immunosuppressant and antiviral agent, and its unique mechanism of action effective against even multi-drug-resistant variants, leflunomide holds great promise in ultimately helping to alleviate the clinical struggle to balance graft-preserving immunosuppression with control of CMV disease.

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1988, p. 997–1001
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Vol. 32, No. 7

Exhibit B

Uridine Reverses the Toxicity of 3′-Azido-3′-Deoxythymidine in Normal Human Granulocyte-Macrophage Progenitor Cells In Vitro without Impairment of Antiretroviral Activity

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We evaluated the effects of natural purine and pyrimidine nucleosides on protection from or reversal of 3′-azido-3′-deoxythymidine (AZT) cytotoxicity in human bone marrow progenitor cells by using clonogenic assays. The selectivity of the "protection" or "rescue" agents was examined in evaluating the antiretroviral activity of AZT in combination with these modulating agents and of AZT alone. Following exposure of human granulocyte-macrophage progenitor cells for 2 h to 5 μM AZT (70% inhibitory concentration), increasing concentrations of potential rescue agents were added. Cells were cultured, and colony formation was assessed after 14 days. At concentrations of up to 50 μM no natural 2′-deoxynucleosides, including thymidine, were able to reverse the toxic effects of AZT. Dose-dependent reversal was observed with uridine and cytidine, and essentially complete reversal was achieved with 50 μM uridine. In the protection studies, 100 μM thymidine almost completely antagonized the inhibition of granulocyte-macrophage colony formation produced by 1 μM AZT (50% inhibitory concentration), and 50 μM uridine effected 60% protection against a toxic concentration of AZT (5 μM) (70% inhibitory concentration). The antiretroviral activity of AZT in human peripheral blood mononuclear cells, assessed by reverse transcriptase assays, was substantially decreased in the presence of thymidine, whereas no impairment of suppression of viral replication was observed in the presence of uridine in combination with AZT at a molar ratio (uridine/AZT) as high as 10,000. This demonstration of the capacity of uridine to selectively rescue human bone marrow progenitor cells from the cytotoxicity of AZT suggests that use of uridine rescue regimen with AZT may have potential therapeutic benefit in the treatment of acquired immunodeficiency syndrome.

3′-Azido-3′-deoxythymidine (AZT), a pyrimidine nucleoside synthesized two decades ago by Horwitz et al. (7), has recently been shown to transiently improve certain immunological functions in some patients with acquired immunodeficiency syndrome (AIDS) (3), resulting in a decrease in the incidence of opportunistic infections and prolonging survival. The antiretroviral effects of AZT are probably based upon its conversion through cellular kinases to AZT triphosphate, which binds to reverse transcriptase and thereby inhibits viral DNA synthesis by chain termination (4). Although AZT selectively inhibits the replication of human immunodeficiency virus type 1 (HIV) (10), its applications in preliminary clinical trials (11, 15) were limited by expressions of bone marrow toxicity. Consistent with these expressions we recently reported (12) that continuous exposure to AZT for 14 days effected a dose-dependent inhibition of human granulocyte-macrophage CFU (CFU-GM) and erythroid burst-forming unit colonies, the 50% inhibitory concentrations being 0.9 ± 0.1 and 2.4 ± 0.4 μM for the respective colonies. Several pharmacologic approaches are potentially available to improve the chemotherapeutic selectivity of AZT. Such an improvement can be theoretically obtained with synergistic combination chemotherapy, as recently demonstrated with recombinant human granulocyte-macrophage colony-stimulating factor (5) and alpha A interferon (6), potentially allowing a reduction in AZT concentrations. Selective "protection" or "rescue" combinations may also achieve this goal. In this approach, the modulating agents are used at a time and a dosage that counteract (protection) or reverse (rescue) the toxic effects in the host cell without interfering with the chemotherapeutic activity. This concept has been previously used in cancer chemotherapy with methotrexate and its "antidote," leucovorin (2), and more recently for treating protozoan infections with trimetrexate and leucovorin in AIDS patients (1).

The present report relates the results of an evaluation of the capacities of various natural nucleosides to protect or to reverse AZT toxicity in human host cells. The selectivity of the metabolic modulations was assessed by evaluating their antiretroviral activity in comparison with that of AZT alone in HIV-infected cells.

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MATERIALS AND METHODS

Chemicals. Purine and pyrimidine ribonucleosides and deoxyribonucleosides were purchased from Sigma Chemical Co., St. Louis, Mo. AZT was synthetized in our laboratory by the procedure of Lin and Prusoff (8) and had a purity of >99%, as assessed by high-pressure liquid chromatography. The structure of the compound was confirmed by proton nuclear magnetic resonance, ^{13}C nuclear magnetic resonance, and infrared spectroscopy. Other chemicals were of the highest quality commercially possible.

Virus strains. HIV strain LAV was obtained from the

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Centers for Disease Control, Atlanta, Ga., and propagated as recently described (13).

Preparation of cells. Human bone marrow cells were collected by aspiration from the posterior iliac crest of normal healthy volunteers and treated with heparin, and the mononuclear population was separated by Ficoll-Hypaque gradient centrifugation. Cells were washed twice in Hanks balanced salt solution and counted with a hemacytometer, and their viability was >98%, as assessed by trypan blue exclusion. Peripheral blood mononuclear (PBM) cells were obtained from the whole blood of healthy HIV- and hepatitis B virus- seronegative volunteers and collected by single-step Ficoll-Hypaque discontinuous gradient centrifugation.

Assay of CFU-GM for drug cytotoxicity and rescue or protection studies. The culture assay of CFU-GM was performed by a bilayer soft-agar method as recently described (12). McCoy 5A nutrient medium supplemented with 15% dialyzed fetal bovine serum (heat inactivated at 56°C for 30 min) (GIBCO Laboratories, Grand Island, N.Y.) was used in all experiments. This medium completely lacked thymidine and uridine.

In the rescue studies, mononuclear cells (10^5/ml) were exposed for 2 h at 37°C in 5 ml of McCoy 5A nutrient medium to 5 μM AZT, corresponding to a 70% inhibitory concentration. At the end of the 2-h incubation period, cells were washed twice with fresh cold incubation medium to wash out the AZT. Cells were subsequently cloned in 0.3% agar in the presence of increasing concentrations of the modulating compound or in medium alone (control). After 14 days of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air, colonies (≥50 cells) were counted by using an inverted microscope.

In the protection studies, AZT (1 or 5 μM) and either medium (control) or various concentrations of thymidine or uridine were added simultaneously. Cells were exposed continuously under these conditions for 14 days, and colonies (≥50 cells) were then scored. The toxicity of each purine and pyrimidine analog investigated in these studies (see Table 1) was assessed by continuous exposure for 14 days by the same technique.

Anti-HIV assay based on reverse transcriptase activity. After phytohemagglutinin stimulation for 3 days, PBM cells (5×10^5/ml) were infected with HIV strain LAV at a concentration of about 100 50% tissue culture infective doses per ml and cultured in the presence of various concentrations of AZT alone or in combination with either uridine or thymidine. The virus was allowed to adsorb for 45 min, and then drugs (AZT and uridine or thymidine) were added. A virus control (no drug) and a cell control (no virus or drug) were also included. On day 5 after infection, clarified supernatant fluids were centrifuged in a Beckman 70.1 Ti rotor at 40,000 rpm for 30 min. The concentrated, disrupted virus was subjected to reverse transcriptase assays as recently described by Spira et al. (13). Antiretroviral efficacy was determined by calculating the percent reduction in reverse transcriptase activity observed in drug-treated, virus-infected cultures as compared with enzyme activity in virus-infected control cultures.

RESULTS

Effect of a short exposure (2 h) of normal human bone marrow progenitor cells to AZT on colony growth. Initial experiments were designed to establish the concentration dependence of human bone marrow progenitor cell toxicity produced by AZT after 2 h of drug exposure. Normal human bone marrow cells were incubated at 37°C for 2 h with various concentrations of AZT, and cells were washed twice prior to plating. Cell viability was determined by soft-agar cloning and measurement of colony formation after drug treatment as described in Materials and Methods. After 2 h of drug exposure, the toxic effects of AZT (Fig. 1) were quite similar to those recently reported for these cells after continuous exposure (14 days) to AZT (12), suggesting that the toxicity of AZT in human bone marrow progenitor cells in vitro is probably mediated through early effects.

Ability of purine or pyrimidine derivatives to reverse the toxicity of AZT in human bone marrow cells. The effects of

TABLE 1. Reversal of AZT cytotoxicity in human bone marrow progenitor cells by naturally occurring purine and pyrimidine nucleosides

Compound and concn (μM)	Survival (% of untreated control)[a] in the presence of compound:	
	Alone	With AZT (5 μM)[b]
Thymidine		
0	100	22.8 ± 7.8
5	ND[c]	24.6 ± 7.1
10	85.4 ± 3.1	24.6 ± 8.4
50	84.7 ± 14.1	17.7 ± 7.5
Cytidine		
0	100	22.8 ± 7.8
5	ND	25.3 ± 12.4
10	94.0 ± 7.3	23.5 ± 5.3
50	93.3 ± 6.2	50.0 ± 14.0[d]
Uridine		
0	100	22.8 ± 7.8
5	ND	24.5 ± 13.6
10	85.4 ± 3.1	43.2 ± 14.4[e]
50	84.7 ± 14.1	100.7 ± 20.3[d]
100	ND	85.6 ± 10.4[d]
2′-Deoxyuridine		
0	100	33.0 ± 16.0
5	ND	37.0 ± 10.4
10	100 ± 8.0	34.0 ± 6.0
50	91.3 ± 2.3	35.0 ± 10.5
2′-Deoxyadenosine		
0	100	33.0 ± 16.0
5	ND	36.0 ± 7.0
10	66.7 ± 5.5	37.3 ± 15.5
50	63.3 ± 6.4	42.3 ± 8.1
2′-Deoxyguanosine		
0	100	33.0 ± 16.0
5	ND	33.6 ± 16.2
10	83.4 ± 3.8	30.0 ± 6.2
50	ND	40.0 ± 6.0
2′-Deoxycytidine		
0	100	33.0 ± 16.0
5	ND	28.0 ± 7.0
10	82.7 ± 12.6	34.0 ± 10.4
50	94.6 ± 4.6	35.0 ± 4.6

[a] Each value represents the mean ± standard deviation in at least three experiments with at least three different marrow donors.
[b] Cells were incubated with AZT (5 μM) for 2 h, washed twice, and cultured for clonal growth for 2 weeks in the presence of purine or pyrimidine analogs.
[c] ND, Not determined.
[d] $P < 0.001$ as compared with the control.
[e] $P < 0.01$ as compared with the control.

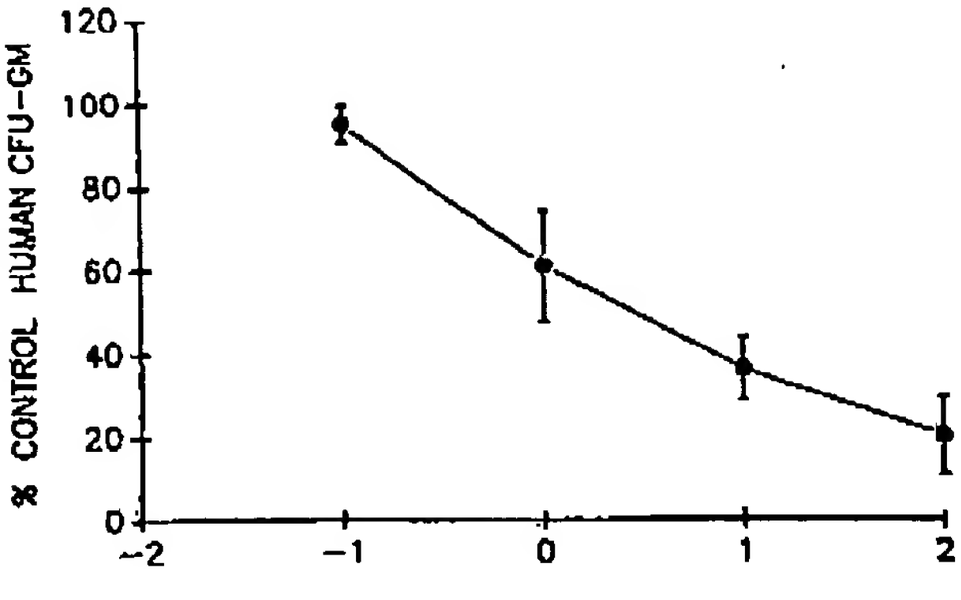

FIG. 1. Effects of a short exposure (2 h) of normal human bone marrow progenitor cells to increasing concentrations of AZT. Each point represents the mean ± standard deviation of at least six experiments with different marrow donors.

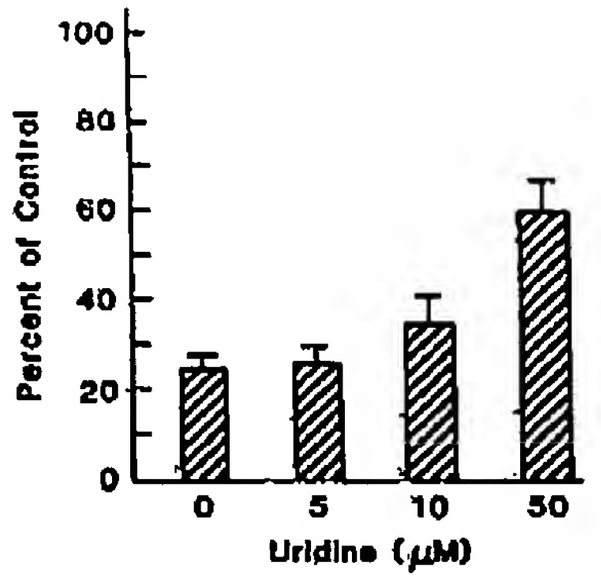

FIG. 2. Effect of AZT (5 μM) on hematopoietic colony growth by normal human bone marrow progenitor cells in the presence of various concentrations of uridine. Columns represent the mean percentage of inhibition of CFU-GM colonies in three separate experiments; bars represent the standard deviation. The mean number of colonies in the control plates (without AZT and uridine) was 60 ± 5 CFU-GM per 10^5 cells. P was <0.01 for 10 μM uridine and <0.001 for 50 μM uridine as compared with the control.

adding purine or pyrimidine nucleoside analogs to human hematopoietic progenitor cells following 2 h of exposure to 5 μM AZT (70% inhibitory dose) are shown in Table 1. No natural purine or pyrimidine 2′-deoxynucleosides (2′-deoxyuridine, 2′-deoxyadenosine, 2′-deoxyguanosine, 2′-deoxycytidine, and thymidine) up to a concentration of 50 μM reversed the toxic effects of AZT, nor was reversal achieved with 2′-deoxynucleoside concentrations of up to 200 μM (data not shown). In contrast, nontoxic concentrations of uridine or cytidine effected a significant and dose-dependent reversal of AZT toxicity. Essentially complete reversal was achieved with 50 μM uridine, and no significant difference was detected in the presence of higher concentrations of uridine (100 μM). At concentrations of 50 μM the rescue effect of cytidine was less than that of uridine. The reversal of AZT toxicity by cytidine probably depends on the conversion of cytidine to uridine by cytidine deaminase, a requirement that could explain the difference in the rescue potencies of the two pyrimidine derivatives.

Protection from AZT toxicity by uridine in human bone marrow cells. Simultaneous exposure to 5 μM AZT (70% inhibitory dose) and various concentrations of uridine was also investigated in our studies to assess whether uridine could protect human bone marrow progenitor cells from AZT toxicity when both drugs were present throughout the experiment. Figure 2 illustrates the effects of 5 μM AZT on hematopoietic colony growth of normal human bone marrow progenitor cells in the presence of 5 to 50 μM uridine. Dose-dependent protection was observed, with 50 μM uridine effecting approximately 60% protection in the presence of a toxic concentration of AZT (5 μM).

Protection from AZT toxicity by thymidine in human bone marrow cells. Since thymidine counteracts the antiretroviral activity of AZT in ATH8 cells (10) and both AZT and thymidine appear to share the same activating enzymes (i.e., thymidine and thymidylate kinases) (4), protection from AZT toxicity by thymidine was investigated in human bone marrow progenitor cells. Human granulocyte-macrophage precursor cells, grown in soft agar, were exposed continuously for 14 days to 1 μM AZT (50% inhibitory dose) and 10 to 100 μM thymidine. The latter agent antagonized the inhibition of colony formation by AZT in a dose-dependent manner, almost complete protection being effected by 100 μM thymidine (Fig. 3).

Effect of thymidine and uridine on the antiretroviral activity of AZT in HIV-infected human PBM cells. The effects of thymidine and uridine on the capacity of AZT to inhibit HIV replication in human PBM cells was evaluated (Table 2). Essentially full protection against HIV production was achieved with AZT alone at a concentration of approximately 0.01 μM, in agreement with recently published data (9). The presence of thymidine led to a substantial loss of the antiretroviral activity of AZT, as reported previously (10). A concentration of 10 μM thymidine reduced the inhibition of HIV by 0.01 μM AZT by approximately 50%, and the anti-HIV activity of 0.01 μM AZT was essentially abolished by 100 μM thymidine. In contrast, concentrations of uridine of up to 100 μM and in combination with AZT at a molar

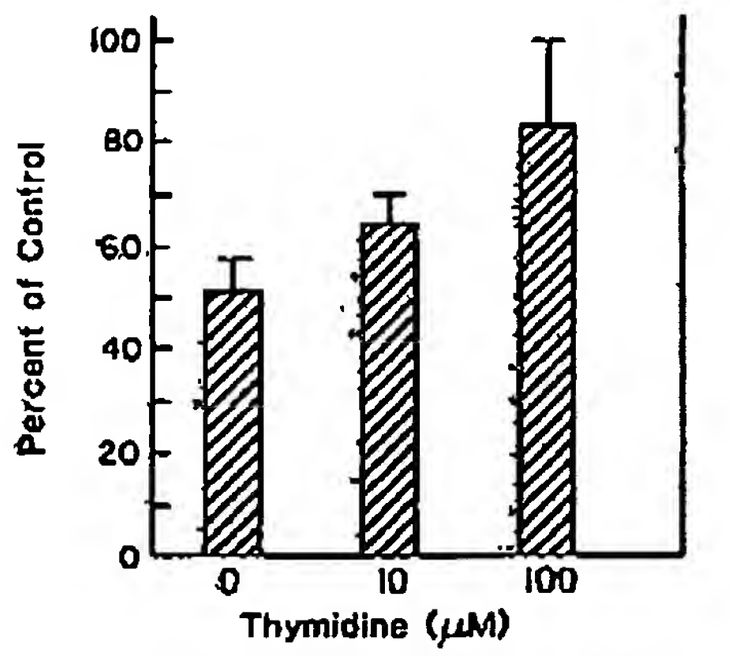

FIG. 3. Effect of AZT (1 μM) on hematopoietic colony growth by normal human bone marrow progenitor cells in the presence of various concentrations of thymidine. Columns represent the mean percentage of inhibition of CFU-GM colonies in three separate experiments; bars represent the standard deviation. The mean number of colonies in the control plates (without AZT and thymidine) was 80 ± 20 CFU-GM per 10^5 cells.

TABLE 2. Effect of thymidine and uridine on AZT antiretroviral activity in HIV-infected human PBM cells

Treatment and concn (μM)	% Inhibition on day 5[a] (corrected)
AZT	
0.001	0
0.01	81.9
0.1	96.6
Thymidine	
10	0
100	0
Uridine	
10	7.3
100	5.1
AZT-thymidine	
0.01-0.1	81.4
0.01-1	85.0
0.01-10	52.6
0.01-100	4.1
AZT-uridine	
0.01-10	75.1
0.01-100	63.2
0.1-10	96.7
0.1-100	96.1

[a] The mean of triplicate counts (± standard deviation) for the virus control was 234,780 ± 26,600 dpm/ml (equivalent to an approximate incorporation of 3.6 pmol of dTMP into the acid-insoluble product). Counts for the blank and negative control (no virus or drug) were 355 and 1,535 dpm, respectively. A positive control for the reverse transcriptase assay was also included (count, 313,000 dpm).

ratio (uridine/AZT) as high as 10,000 had no effect on the antiretroviral activity of AZT. Cell viability and growth, as assessed by trypan blue exclusion and with a Coulter Counter, were similar among the cultures, and no toxicity was observed in the presence of 100 μM uridine (Table 3).

TABLE 3. Effect of AZT and uridine alone or in combination on human PBM cell growth and cell viability[a]

Treatment and concn (μM)	Growth (% of untreated control)	Viability (% of untreated control)
AZT		
0.1	102.9	113.2
1	100	90.6
10	81.2	100
100	79.4	103.1
Uridine		
1	100	109.4
10	94.1	100
100	87.0	100
AZT-uridine		
1-1	97.1	78.8
1-10	105.9	72.5
1-100	78.1	78.2

[a] The mean numbers of human PBM cells (± standard deviation) in the cell growth and viability studies were (3.2 ± 0.6) × 10^6 cells per ml and 3.4 ± 0.3 cells per ml, respectively. Cells were stimulated with phytohemagglutinin for 2 days and subsequently exposed for 5 days to various concentrations of AZT or uridine alone or in combination.

These data suggest that, unlike thymidine, uridine does not interfere with the uptake and/or metabolic activation of AZT in HIV-infected human PBM cells or any other mechanism(s) by which AZT inhibits HIV replication.

DISCUSSION

In a previous report from this laboratory, we demonstrated that AZT directly suppressed human hematopoietic colony growth in a dose-dependent manner by direct interaction with CFU-GM and erythroid burst-forming unit progenitor cells (12). These findings were consistent with the observation that anemia and neutropenia were the major adverse effects of AZT administration to AIDS patients (11, 15). In an effort to obviate this untoward effect of AZT, we have sought to determine in the present study whether natural nucleosides can protect against or reverse the toxicity of AZT in human bone marrow progenitor cells without affecting the antiretroviral activity of AZT. As reported here, thymidine can counteract or protect against the toxicity of AZT in normal human bone marrow cells, but it also antagonizes the antiretroviral activity of AZT (Table 2). These data probably reflect a decrease in the formation of AZT triphosphate, since both AZT and thymidine utilize the same activating enzymes (i.e., thymidine and thymidylate kinases) to exert their pharmacologic action. It is particularly important that thymidine, even at concentrations of up to 100 μM, was not able to reverse the toxic effects of 5 μM AZT (70% inhibitory concentration) for human granulocyte-macrophage precursor cells. This result suggests that the administration of thymidine sequentially with AZT in vivo probably will not prevent its toxic effects, as previously speculated (11).

Our experiments also showed that the hematopoietic toxicity of AZT was consistently reversed by uridine and to a lesser extent by cytidine. The inhibition of CFU-GM colony formation at an AZT concentration of 5 μM was essentially reversed when 50 μM uridine was added to the cultures. The percentage of rescue of CFU-GM colony formation was proportional to the concentration of uridine, suggesting that the reversal was a competitive process. The same concentration of cytidine only partially reversed the toxic effects of AZT on colony formation. In these studies, cytidine probably acted through its conversion to uridine by cytidine deaminase, explaining the quantitative difference in the rescue between the two pyrimidine derivatives. Uridine was shown also to protect normal human bone marrow progenitor cells from AZT toxicity, and 60% protection was achieved when cells were exposed to both 5 μM AZT and 50 μM uridine for 14 days.

No difference in the inhibition of viral replication was observed in HIV-infected PBM cells when uridine was combined with AZT at different molar ratios as compared with AZT alone. Therefore, the combination of AZT and uridine appears to selectively reverse the hematopoietic effects of AZT without decreasing its antiretroviral activity. Uridine pharmacokinetic and toxicity studies have been recently reported in humans (14), and "rescuing" concentrations of uridine may be achieved in vivo, with a tolerable toxicity, making this combination potentially suitable for the treatment of AIDS. A potential mechanism(s) which may account for the reversal of or protection from AZT cytotoxicity by uridine in human bone marrow cells is unclear, and further investigations are currently in progress.

In summary, the high degree of selectivity of the uridine rescue between human bone marrow progenitor cells and

HIV-infected cells suggests that the combined use of AZT and uridine may be of importance in the treatment of AIDS. Although the results of the present in vitro studies must be cautiously extended to the clinical situation, the possible use of uridine for rescue may have a potential therapeutic benefit in that the antiretroviral activity of AZT is not affected while the host toxicity of AZT is minimized. This novel strategy for modulating AZT therapy deserves further biochemical and/or pharmacologic investigations, which may lead to carefully controlled clinical evaluations.

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Exhibit C.

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Uridine in the prevention and treatment of NRTI-related mitochondrial toxicity.

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Long-term side effects of antiretroviral therapy are attributed to the mitochondrial (mt) toxicity of nucleoside analogue reverse transcriptase inhibitors (NRTIs) and their ability to deplete mtDNA. Studies in hepatocytes suggest that uridine is able to prevent and treat mtDNA depletion by pyrimidine NRTIs [zalcitabine (ddC) and stavudine (d4T)] and to fully abrogate hepatocyte death, elevated lactate production and intracellular steatosis. Uridine was also found to improve the liver and haematopoietic toxicities of zidovudine (AZT), which are unrelated to mtDNA depletion, and to prevent neuronal cell death induced by ddC. Most recently, uridine was found to prevent the onset of a lipoatrophic phenotype (reduced intracellular lipids, increased apoptosis, mtDNA depletion and mt depolarization) in adipocytes incubated long-term with d4T and AZT. Various steps of mt nucleoside utilization may be involved in the protective effect, but competition of uridine metabolites with NRTIs at polymerase y or other enzymes is a plausible explanation. Pharmacokinetic studies suggest that uridine serum levels can be safely increased in humans to achieve concentrations which are protective in vitro (50-200 microM). Uridine was not found to interfere with the antiretroviral activity of NRTIs. Mitocnol, a sugar cane extract which effectively increases uridine in human serum, was beneficial in individual HIV patients with mt toxicity and is now being tested in placebo-controlled randomized trials. Until these data become available, the risk-benefit calculation of using uridine should be individualized. The current safety data justify the closely monitored use of uridine in individuals who suffer from mt toxicity but who cannot be switched to less toxic NRTIs.

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IN VIVO ACTIVITY OF LEFLUNOMIDE

PHARMACOKINETIC ANALYSES AND MECHANISM OF IMMUNOSUPPRESSION

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Background. Leflunomide is an experimental drug with demonstrated ability to prevent and reverse acute allograft and xenograft rejection. The two biochemical activities reported for the active metabolite of leflunomide, A77 1726, are inhibition of tyrosine phosphorylation and inhibition of dihydroorotate dehydrogenase, an enzyme necessary for *de novo* pyrimidine synthesis. These activities can be distinctly separated *in vitro* by the use of uridine, which reverses the anti-proliferative effects of A77 1726 caused by inhibition of *de novo* pyrimidine synthesis. We report the effect of uridine on the *in vivo* immunosuppressive activities of leflunomide.

Methods. We first quantified the serum levels of A77 1726, the active metabolite of leflunomide, after a single treatment of leflunomide (5, 15, and 35 mg/kg). Additionally, we quantified the levels of serum uridine and of nucleotide triphosphates in the liver, spleen, and lymph nodes of Lewis rats after the administration of a single dose of uridine (500 mg/kg; i.p.). Lewis rats heterotopically transplanted with brown Norway or Golden Syrian hamster hearts were treated for 50 or 75 days with leflunomide (5, 15, and 35 mg/kg/day, gavage) alone or in combination with uridine (500 mg/

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kg/day; i.p.). Hematocrits were determined and the levels of alloreactive or xenoreactive immunoglobulin (IgM and IgG were determined by flow cytometric analysis. The allograft and xenografts, small bowel, liver, kidney, and spleen were subjected to pathological examination.

Results. A linear relationship was observed between the serum A77 1726 concentrations in Lewis rats and the dose of leflunomide administered. Peak A77 1726 concentrations were 20.9, 71.8 and 129.3 mg/l (77.5, 266.1 and 478.8 μM) for the 5, 15, and 35 mg/kg doses of leflunomide, respectively. The concentration of uridine in the serum of normal Lewis rats is 6.5 μM; after i.p. administration of 500 mg/kg uridine, the serum uridine concentrations peaked at 384.1 μM in 15–30 min. The rapid elimination of uridine was not reflected in the lymphoid compartments, and the pharmacokinetics of pyrimidine nucleotides in the spleen resembled that of A77 1726. This dose of uridine, when administered daily (500 mg/kg/day, i.p.), weakly antagonized the immunosuppressive activities of leflunomide (5, 15, and 35 mg/kg/day) in the allotransplantation model. In contrast, in the xenotransplantation model, the same concentration of uridine completely antagonized the immunosuppressive activities of low-dose leflunomide (15 mg/kg/day) and partially antagonized the immunosuppressive activities of high-dose leflunomide (35 mg/kg/day). Toxicities associated with high-dose leflunomide (35 mg/kg/day) were anemia, diarrhea, and pathological changes in the small bowel and liver. These toxicities were significantly reduced by uridine co-administration.

Conclusion. These studies reveal that the blood levels of A77 1726 in Lewis rats satisfy *in vitro* requirements for both inhibition of *de novo* pyrimidine synthesis and protein tyrosine kinase activity. Our data also illustrate that the *in vivo* mechanism of immunosuppression by leflunomide is complex and is affected by at least the following four factors: type and vigor of the immune response, availability of uridine for salvage by proliferating lymphocytes, species being investigated, and concentration of serum A77 1726.

Leflunomide [N-(trifluoromethylphenyl)-methylisoxazol-4-carboxamide; HWA 486 or SU101] is an experimental immunosuppressive drug with demonstrated ability to prevent and reverse acute allograft and xenograft rejection (reviewed in (1)). Leflunomide is rapidly converted *in vivo* to the active metabolite, A77 1726. The two biochemical activities ascribed to A77 1726 are inhibition of protein tyrosine kinases (2) and of dihyroorotate dehydrogenase (DHO-DHase*), a key enzyme in the *de novo* synthesis of pyrimidine nucleotides (3–6). The significantly lower IC_{50} required *in vitro* to inhibit DHO-DHase relative to tyrosine kinases has led many researchers in this field to suggest that the *in vivo* antiproliferative and immunosuppressive activities of leflunomide, and its active metabolite, A77 1726, result from the inhibition of the enzymatic activity of DHO-DHase (1).

Several lines of evidence suggest that this hypothesis may not be supported *in vivo*. First, the serum uridine in human and rodents (5–15 μM (7, 8)) could be converted to pyrimidine nucleotides by the salvage pathway, resulting in a normalization of intracellular pyrimidine nucleotide levels, despite inhibition of *de novo* pyrimidine synthesis. Second, when A77 1726 is used *in vitro* at concentrations >50 μM, the antiproliferative activity on T cells and B cells could no longer be completely reversed by the addition of exogenous uridine, suggesting a second mode of activity at these concentrations that is independent of pyrimidine depletion (*9*, *10*). Third, patients with a genetic defect in *de novo* pyrimidine synthesis, hereditary orotic aciduria, do not have undue susceptibility to infection, indicating that they are not significantly immunosuppressed (reviewed in (*11*)). *In vitro* cellular immune defects have been reported in some of these patients; however, these immune defects can be attributed to lymphopenia arising from a generalized defect in hematopoiesis, rather than to an intrinsic inability of T cells to proliferate in response to antigen stimulation (*12*, *13*).

These observations prompted us to examine whether the immunosuppressive activities of leflunomide in rats and mice are mediated by the inhibition of *de novo* pyrimidine synthesis. *In vitro* studies suggest that the effects of A77 1726 that are caused by inhibition of *de novo* pyrimidine synthesis can be reversed with uridine. Using a similar approach, we report the effects of uridine on the *in vivo* immunosuppressive activities and toxic side effects of leflunomide in mice and rats.

MATERIALS AND METHODS

Extraction and quantitation of A77 1726 from serum. For single-dose pharmacokinetics, Lewis rats not receiving transplants were treated with a single dose of 5, 15, or 35 mg/kg/day leflunomide by gavage. Serum was collected at the indicated times and stored at 70°C before extraction. Serum, 50 μl, was mixed with 25 μl 4'-(trifluoromethyloxy)-acetanilide (TFMO; Sigma, St. Louis, MO), 200 μl 0.5 M HCl, and 4 ml extraction solvent (1:1 pentane and dichloromethane; Sigma). The mixture was vortexed for 1 hr, then centrifuged at 2000 *g* for 5 min (Centra-8, IEC, Needham Heights, MA). After freezing the mixture for 1 hr at −20°C, the organic phase was decanted into a new tube and dried under a hood at room temperature. Then 200 μl of reconstituting solution (1:1 acetonitrile and water) was added, and the mixture was vortexed and centrifuged at 2000 g for 2 min. Finally, 65 μl of the mixture was analyzed by high-performance liquid chromatography (HPLC) (Waters, Milford, MA), using a Symmetry C18 column (4.6 × 250 mm; Waters). A77 1726 was separated with a mobile phase composed of 50% acetonitrile and 50% buffer (25 mM KH_2PO_4). The corresponding peak of A77 1726 was compared with a standard of purified A77 1726 (a gift from Robert R. Bartlett, Hoechst Marion Roussel, Wiesbaden, Germany), and the concentrations were calculated on the basis of a standard curve of purified A77 1726.

Extraction and quantitation of uridine from serum. For single-dose pharmacokinetics, Lewis rats not receiving transplants were treated with a single dose of uridine. Serum was collected at the indicated times and stored at 70°C before extraction. Serum samples were diluted two fold in 0.9% NaCl, and uridine was extracted by the addition of an equal volume of 0.8 M trichloric acid and then neutralized with an equal volume of 0.5 M tri-n-octylamine in Freon. Serum uridine was detected by HPLC, using a Lichrosorb-10RP-18 column (Whatman, Alltech, Deerfield, IL) and an elution solution (5 mM KH_2PO_4, pH 3.8), at a flow of 1 ml/min. The uridine peak was identified by its retention time and spectrum compared with a uridine standard (Sigma). Uridine concentrations were calculated on the basis of a standard curve.

Extraction and quantitation of nucleotide triphosphate from tissues. For single-dose pharmacokinetic analysis, normal Lewis rats were treated with a single dose of uridine. The spleen, lymph nodes,

* Abbreviations used: DHO-DHase, dihydroorotate dehydrogenase; Ig, immunoglobulin; HPLC, high-performance liquid chromatography; Ka, rate of absorption; Ke, rate of excretion; PCV, packed cell volume.

and liver (100 mg tissue) were homogenized and nucleotide triphosphates were extracted with 0.4 M trichloric acid and neutralized with an equal volume of 0.5 M tri-n-octylamine in Freon 113, as previously described (*9*, *14*). Nucleotides were separated using a Whatman anion exchange column (Particil 10 SAX, Alltech) and a linear gradient elution of potassium phosphate buffer, pH 4.5 (10–500 mM). The corresponding peaks of four nucleotides were detected by HPLC (Waters), and the concentrations were calculated on the basis of a standard curve of purified nucleotides (Sigma).

Pharmacokinetic and statistical analysis. The pharmacokinetic analysis of serum A77 1726, uridine, and tissue UTP levels were conducted using a nonlinear regression analysis with a Gaussian algorithm. The time-concentration data were fitted to the open, one-compartment, extravascular model:

$$C_t = C^\circ(\exp(-K_e{}^*t) - \exp(-K_a{}^*t))$$

where C_t, C°, K_e, K_a, and t are the serum concentration at time t, the theoretical initial concentration, the excretion constant, the absorption constant, and the time after drug administration. The best fit values for C°, K_e, and K_a were used to calculate the terminal half-life ($T_{1/2}$) using the formula: $T_{1/2}=0.693/K_e$. The area under the curve was calculated using the trapezoidal method. Statistical differences between pharmacokinetic parameters were analyzed using a *t* test or analysis of variance.

Transplantation model and drug treatment. Lewis or brown Norway rats, and Golden Syrian hamsters were purchased from Harlan Labs (Indianapolis, IN). Balb/c and C3H mice were purchased from Jackson Labs (Bar Harbour, ME). Heart grafts were heterotopically transplanted into the abdomen of the recipients after a modified protocol described by Ono *et al.* (*15*). Leflunomide (5–35 mg/kg/day, custom synthesized for research purposes) was suspended in 1% carboxymethyl cellulose and administered by gavage. Uridine (Sigma) was dissolved in 0.9% NaCl for daily i.p. injections. The transplanted hearts were monitored daily, and rejection was defined as the complete cessation of pulsations in the transplanted heart.

Packed cell volume. Rats were bled every 2 weeks or on the day they were killed, through the orbital vein, using a microhematocrit capillary tube (Baxter, Deerfield, IL). The blood was centrifuged for 15 mins at 650 g, and the percentage of packed cell volumes was determined with a micro-hematocrit capillary tube reader (Critocaps, Oxford Lab).

Quantification of allo-specific and hamster-specific IgM and IgG titers. Quantification of allo-specific or hamster-specific antibodies was performed, as previously described (*16*, *17*). Lymphocytes (5×10^6) from lymph nodes isolated from brown Norway rats or erythrocytes (10^6) from Golden Syrian hamster were incubated with diluted, heat-inactivated test serum or control naive Lewis rat serum (1:20 dilution) for 30 min at 4°C. Lymphocytes were washed with phosphate-buffered saline, and erythrocytes were washed in 4% (weight/volume) sodium citrate/phosphate-buffered saline. The cells were then stained with phycoerythrin-conjugated F(ab')2 anti-rat immunoglobulin (Ig)M or fluorescein isothiocyanate-conjugated F(ab')2 anti-rat IgG (Jackson ImmunoResearch, West Grove, PA). After staining, the erythrocytes and lymphocytes were washed, fixed in 1% formalin, and analyzed using a flow cytometer (Ortho Cytoron Absolute, Ortho Diagnostic Systems, Raritan, NJ).

Histology and immunohistochemistry. Sections of the spleen, liver, kidney, and small bowel were collected, imbedded in frozen tissue matrix CO.C.T. compound (Sakura Finetek U.S.A., Torrance, CA), and snap-frozen in liquid nitrogen. Sections of these tissues, 5 μm, were made and fixed in 10% formalin. These sections were then stained in hematoxylin and eosin solutions. Allografts were scored according to a modified cardiac biopsy grading by Billingham *et al.* (*18*). Grade 0 (no acute rejection) indicates no evidence of acute rejection or myocyte damage; Grade 1A (focal, mild acute rejection) indicates focal, perivascular, or interstitial infiltrate of mononuclear cells with no myocyte damage; Grade 1B (diffuse, mild, acute rejection) indicates a more diffuse, perivascular or interstitial infiltrate of mononuclear cells with no myocyte damage; Grade 2 (focal, moderate acute rejection) indicates a few focal aggressive inflammatory infiltrate with focal myocyte damage; Grade 3A indicates multifocal aggressive inflammatory infiltrate with myocyte damage; Grade 3B indicates diffuse aggressive inflammatory infiltrate with myocyte necrosis; and Grade 4 indicates diffuse aggressive inflammatory infiltrate with myocyte necrosis, hemorrhage, edema, and vasculitis. Sections for immunohistochemical analysis were fixed in cold acetone and stained with monoclonal antibodies against rat IgM, IgG, TCRαβ, and ED1, using a modified ABC method, as previously described (*18*, *19*).

RESULTS

Single dose pharmacokinetics of A77 1726. Lewis rats received by gavage three different doses of leflunomide (5, 15, 35 mg/kg). Sera were harvested from 4–6 individual rats at the indicated times, and the active metabolite of leflunomide, A77 1726, in the serum was extracted and quantified by HPLC. Increasing concentrations of leflunomide resulted in a dose-dependent increase in A77 1726 in the serum (Fig. 1A). A linear relationship was observed between the peak concentrations and the area under the curve, and the dose of A77 1726 administered (Fig. 1B). The A77 1726 peak concentrations were 20.9, 71.8, and 129.3 mg/l (77.5, 266.1, and 478.8 μM) for the 5, 15, and 35 mg/kg doses of leflunomide, respectively (Fig. 1A). These peak concentrations were observed at 6–8 hr (Fig. 1A), and the mean terminal half-life ($T_{1/2}$) ranged from 3.5 to 5.0 hr, irrespective of the administered leflunomide dose (Table 1). The excretion constant (Ke) and absorption constant (Ka) were not statistically significant between treatment groups ($P>0.05$), and the excretion of A77 1726 followed first-order kinetics even at highest dose (Table 1). Similar analyses performed with Balb/c mice given a single, oral dose of leflunomide (35 mg/kg) revealed similar peak A77 1726 concentrations (140 mg/l=514 μM) and Ka, compared with Lewis rats; however, the Ke, was significantly

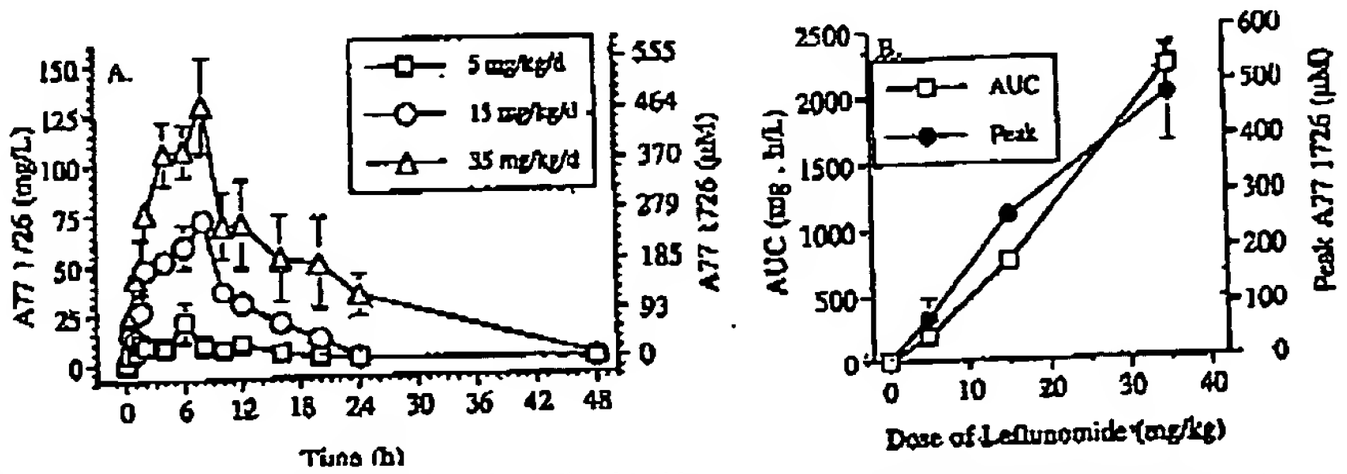

FIGURE 1. (A) Pharmacokinetics of A77 1726 after a single, oral dose of leflunomide in Lewis rats. Serum was harvested at the indicated times, and A77 1726 concentrations determined by HPLC. Data are presented as mean concentrations of 4–6 rats/group, and bars represent SEM. (B) The relationship between the AUC (mg · h/L) and peak concentrations of A77 1726 (μM) and the dose of leflunomide administered.

TABLE 1. Pharmacokinetic parameters of serum A77 1726 following leflunomide administration

Treatment	Ke (ml · min/kg)	Ka (ml · min/kg)	T1/2 (h)	AUC (mg · h/L)
Single-dose leflunomide treatment in Lewis rats				
5 mg/kg leflunomide	0.258±0.112	0.484±0.138	4.42±0.16	183.0±19.6
15 mg/kg leflunomide	0.190±0.005	0.197±0.004	3.49±0.19	764.5±24.0
35 mg/kg leflunomide	0.160±0.023	0.204±0.046	4.97±0.77	2207.3±173.8
Single-dose leflunomide treatment in Balb/c mice				
35 mg/kg leflunomide	0.073±0.032	0.567±0.132	15.02±4.57	3446.5±973.3

lower ($P<0.05$; Table 1) resulting in a longer terminal half live of serum A77 1726.

Single dose uridine pharmacokinetics. *In vitro* and *in vivo* studies have indicated that uridine can be used to counter the effects resulting from the inhibition of *de novo* pyrimidine synthesis (*2–6, 14, 20*). We measured the levels of serum uridine in Lewis rats before and after the administration of a single dose of uridine (500 mg/kg, i.p.). Consistent with previous reports, the mean concentration of uridine in the serum of normal Lewis rats was 6.5±0.9 μM (n=13) (7). A single dose of uridine (500 mg/kg) administered i.p. resulted in a rapid increase in the concentrations of serum uridine. Maximum concentrations were observed within 15–30 min and reached a peak concentration of 384.1±53.5 μM. Serum uridine was rapidly cleared and returned to baseline 4 hr after uridine administration (Fig. 2A).

Intracellular nucleotides following a single dose of uridine. We next determined whether the elevated concentrations of serum uridine resulted in increased intracellular pyrimidine nucleotides in the spleen, lymph nodes, and liver. A cohort of 26 Lewis rats were treated with a single dose of uridine (500 mg/kg, i.p.). The rats were killed after 0, 1, 3, 6, 12, and 24 hr (n=4–5 per group), and lymph nodes and approximately 100 mg of liver and spleen tissue harvested. The nucleotides were extracted from the tissues by trichloroacetic acid, and the concentrations of tissue UTP, CTP, ATP and GTP determined by HPLC. The administration of uridine resulted in 4.1-, 3.6-, 2.4-, and 1.5-fold increases in UTP, CTP, ATP, and GTP, respectively, in the spleen (Fig. 2B–D). The tissue nucleotide concentrations remained at these levels for up to 6 hr and gradually declined to baseline 24 hr after uridine administration. The $T_{1/2}$ of tissue UTP in the spleen was 12.72 hr, with a Ka and Ke of 0.216 and 0.05 ml · min/kg, respectively. Statistical analysis indicated that the pharmacokinetics of UTP levels in the spleen after uridine administration was not significantly different ($P>0.05$) from the pharmacokinetics of A77 1726 after the administration of leflunomide.

After uridine administration, the levels of UTP and CTP increased 4.9- and 2.8-fold, respectively, in the liver tissue and 1.7- and 1.3-fold, respectively, in lymph node cells (Fig. 2B–D). The levels of ATP and GTP were also elevated 3.1- and 2.9-fold, respectively, in the liver and 1.5- and 1.3-fold in the lymph node cells after uridine administration (data not shown). We do not have an explanation for the concomitant increase in ATP and CTP levels but speculate that it could reflect uridine-stimulated increases in metabolic activity in these tissues.

Effect of uridine on the ability of leflunomide to control acute allograft rejection in Lewis rats. Brown Norway hearts transplanted into untreated Lewis rats were rejected in 6–8 days. Treatment with 5 or 15 mg/kg/day of leflunomide resulted in the survival of the allografts for >50 days, whereas

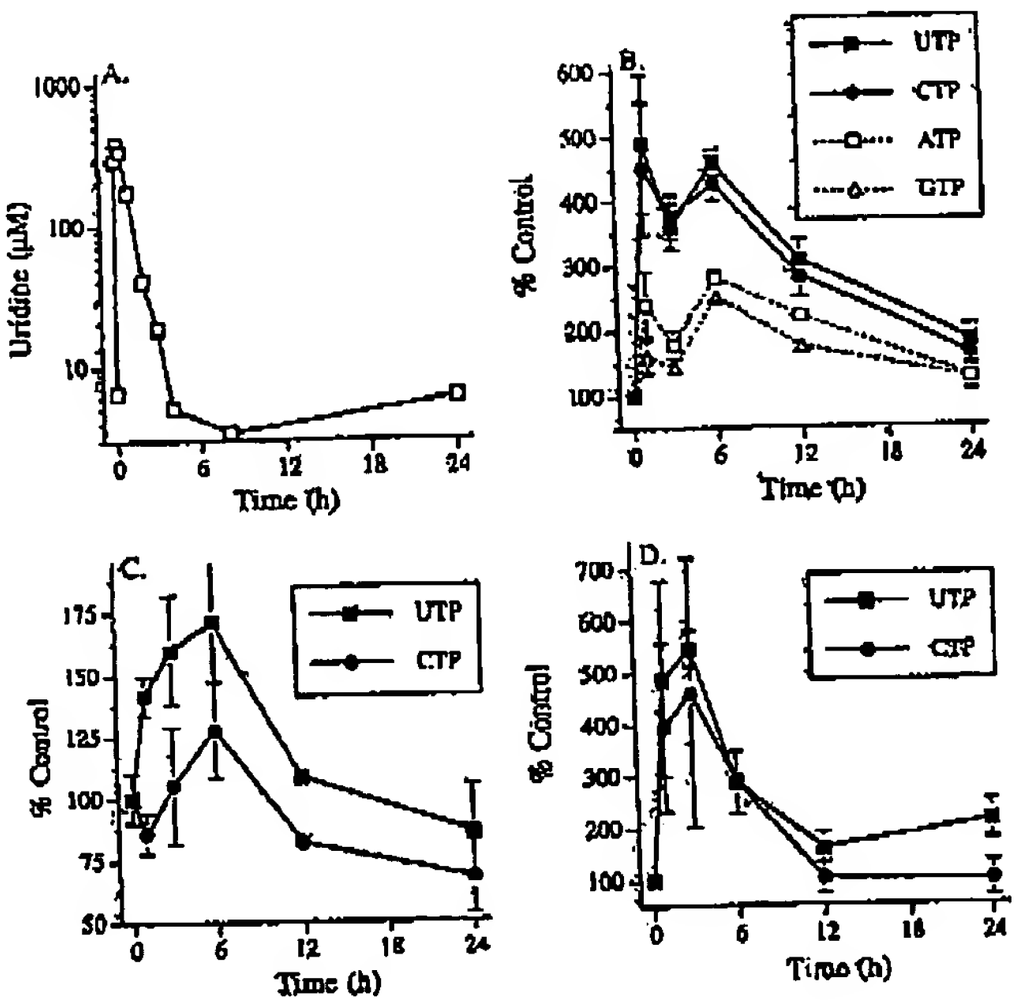

FIGURE 2. Pharmacokinetics of uridine after a single, i.p., dose of uridine (500 mg/kg) in Lewis rats. (A) Serum was harvested at the indicated times and serum uridine concentrations determined by HPLC. Data are presented as mean of 4–13 rats/group, and bars represent SEM. (B–D) Pharmacokinetics of pyrimidine and purine nucleotides in the spleen (B), liver (C) and lymph node (D) after a single, i.p., dose of uridine (500 mg/kg) in Lewis rats. Lewis rats were killed at the indicated times (4–6 rats/group), and the nucleotides extracted following protocols described under Materials and Methods. Data are presented as percentages of untreated controls, and bars represent SEM. The baseline concentrations of UTP, CTP, ATP, and GTP in the liver tissue were 92.0, 4.0, 357.7, 74.8 pg/mg; in the spleen tissue were 28.9, 21.0, 211.8, and 50.9 pg/mg, and in the lymph nodes tissues were 8.8, 7.3, 55.8, and 8.5 pM/20×10^6 cells, respectively.

treatment with 35 mg/kg/day resulted in the sacrifice of all Lewis recipients with beating allografts in <30 days after the transplant because of leflunomide-related toxicities. Uridine (500 mg/kg/day, i.p.) co-administration with leflunomide did not significantly alter allograft survival, and all the hearts were beating on day 50 in the Lewis recipients receiving 5 or 15 mg/kg/day leflunomide plus uridine (Table 2). In the 35 mg/kg/day leflunomide plus uridine group, the toxicity of leflunomide was significantly reduced and 4 of the 5 Lewis recipients were alive, with beating allografts on day 50 after the transplant (Table 2). One of the Lewis recipients in this combination-treatment group died on day 39, with a beating allograft.

TABLE 2. Effect of uridine on the ability of leflunomide to prevent the rejection of allograft (brown Norway) hearts by Lewis rats[a]

Treatment	Allograft survival (d)	Mean (d)	Histological scores
None	6, 6, 7, 7, 7, 7, 8	6.9±0.2	3A–3B (X8)
Lef (5)	>50 (X8)	>50	2, 2, 2, 2, 3A, 3A, 3A, 3B
Lef (5)+Uridine	>50 (X8)	>50	2, 2, 2, 3A, 3A, 3B, 3B, 3B
Lef (15)	>50 (X6)	>50	1A, 1A, 1A, 1A, 1B, 2
Lef (15)+Uridine	>50 (X6)	>50	1A, 1A, 1B, 2, 2, 2
Lef (35)	>13, >26, >28, >29, >29	>25.0±3.0	0, 0, 0, 1A, 1A
Lef (35)+Uridine	>37, >50 (X4)	>47.4±2.6	0, 1A, 1A, 1A, 1A

[a] Uridine was administered i.p. at 500 mg/kg (once a day), whereas leflunomide was administered orally at 5, 15, or 35 mg/kg daily for 50 days. Survival of leflunomide-treated Lewis rats or grafts were calculated from the day of transplantation, and presented as mean±SE. Histological scores were determined when the rats were killed or at the end of the experiment (day 50 after the transplant).

[b] > indicates that Lewis rats died of leflunomide-induced toxicity with beating allografts, or were killed at the end of the experiment (day 50 after the transplant).

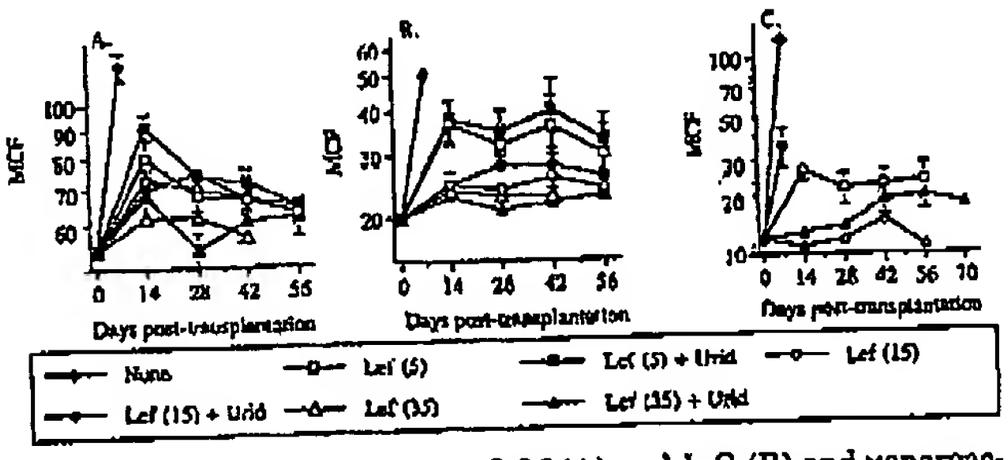

FIGURE 3. Levels of alloreactive IgM (A) and IgG (B) and xenoreactive IgM (C) in Lewis rats treated with leflunomide (Lef 5, 15, or 35 mg/kg/day) alone or in combination with uridine (Urid; 500 mg/kg/day) at the indicated days after the transplant. The relative amounts of alloreactive IgM and IgG and xenoreactive IgM were quantified by flow cytometric analysis, and data are presented as mean channel fluorescence (3 decade log scale)±SE. There were 3–6 rats per group.

We have previously reported that allograft rejection in this model is accompanied by an increase in the titers of alloreactive IgM and IgG (Fig. 3A, B). We here confirm that leflunomide, in a dose-dependent manner, inhibited the increase in both IgM and IgG titers. Co-administration of uridine resulted in a modest increase in the titers of alloreactive IgM and IgG in Lewis rats receiving a 5 mg/kg/day dose of leflunomide but had minimal effects at the higher doses of leflunomide.

On day 50 after the transplant, or at the time the rats were killed, the hearts were harvested and subjected to histological examination. Despite daily treatment with leflunomide (5 mg/kg/day) for 50 days, the allografts demonstrated a mild to moderately intense inflammatory infiltrate and some myocyte necrosis (data not shown). Cellular rejection was significantly reduced in allografts when the recipients were treated with a higher dose of leflunomide (15 mg/kg/day; Fig. 4A, B) and there were no signs of rejection in the allografts harvested from Lewis rats receiving the highest dose of leflunomide (35 mg/kg/day) at the time they were sacrificed (days 13–29 after the transplant; data not shown). In the group receiving uridine (500 mg/kg/day) and leflunomide (5, 15, or 35 mg/kg/day), only slightly exacerbated cellular rejection was observed in the allografts examined on day 50 after the transplant (Table 2, Fig. 4E, F). Some foci of infiltrating T cells and ED1 macrophages were observed, and marginally increased deposition of IgM in the leflunomide plus uridine groups compared to that in the leflunomide (15 mg/kg/day) monotherapy groups (Fig. 4E, F). There was minimal deposition of IgG in the allografts from all leflunomide monotherapy and leflunomide plus uridine groups (data not shown).

Leflunomide induced a dose-dependent reduction in the lymphoid compartments in the spleens of Lewis rats transplanted with Brown Norway hearts. The T lymphocyte zones of the periarteriolar lymphocyte sheath and the B cell zones, located in the follicles, marginal zones, and red pulp (especially around the pulp arterioles) were significantly reduced by leflunomide (15 mg/kg/day; Fig. 4C, D). Uridine completely reversed the effects of leflunomide on the lymphoid compartments in the spleen (Fig. 4G, H), suggesting that, although uridine is able to reverse the antiproliferative effects of leflunomide in the spleen, it has only modest effects on the ability of leflunomide to control allograft rejection.

Effect of uridine on the ability of leflunomide to control acute allograft rejection in C3H mice. Balb/c hearts transplanted into C3H mice were rejected in 8–10 days without immunosuppressive therapy (Table 3). Leflunomide at a dose of 30 mg/kg/day delayed rejection for 21–31 days (31.0±1.8). At this dose, there was no detectable toxicity in the mouse as a result of leflunomide administration. The lack of toxicity probably reflects the reduced sensitivity of mouse DHO-DHase to leflunomide compared with the rat enzyme (2, 3, 21, 22). The mean percent of packed cell volume (PCV) in leflunomide-treated C3H mice, measured on the day of rejection, was 52.3±5.7, while in untreated controls was 52.0±2.8. Co-administration of uridine (500 mg/kg/dose; i.p., twice a day) with leflunomide resulted in a slightly more rapid rate of allograft rejection in 19–31 days (23.8±2.7; N=5). This was the maximum dose of uridine that could be administered in this experimental protocol as 4 of 9 mice died of uridine-related toxicity. The mean percent PCV in the surviving recipients, measured on the day of rejection, was 47.2±4.4%.

Effect of uridine on the ability of leflunomide to control acute xenograft rejection in Lewis rats. The rejection of hamster grafts by Lewis rats is mediated by anti-hamster IgM produced in a T-independent and T-dependent manner (23–25). We have previously reported that leflunomide at 15 mg/kg/day, but not at the 5 mg/kg/day dose, can prevent acute xenograft rejection in the hamster-to-Lewis transplantation model (17). Leflunomide at 15 mg/kg/day resulted in xenograft survival ranging from 48 to ≥76 days (mean graft survival was >63.2±9.7 days). When leflunomide was in-

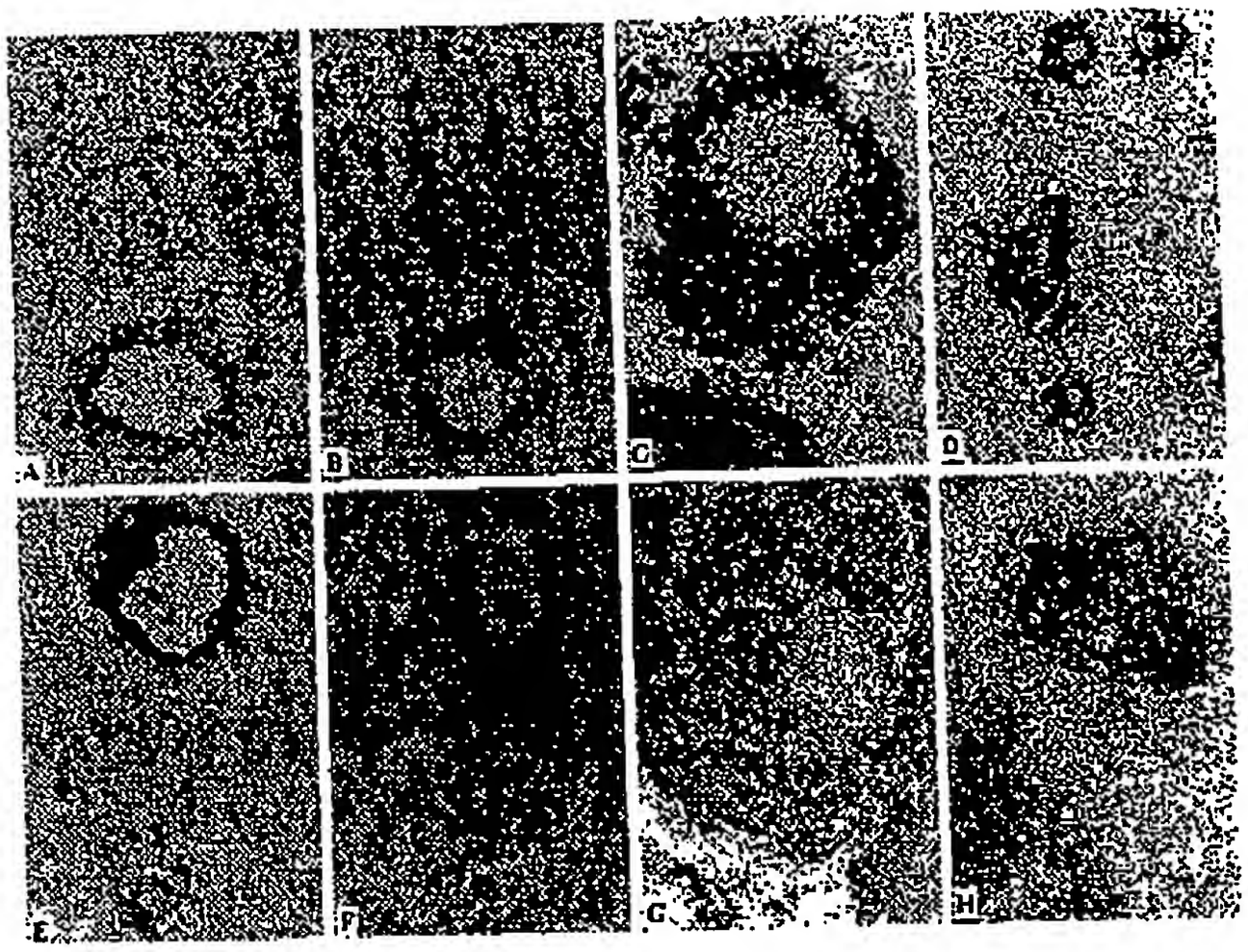

FIGURE 4. The ability of leflunomide (15 mg/kg/day) to inhibit allograft rejection (A and B) was minimally affected by uridine (500 mg/kg/day) co-administration (E and F). The proliferation of T and B cells in the recipient spleen in response to allograft stimulation was inhibited by leflunomide (15 mg/kg/day) (C and D), and this inhibition was reversed by uridine (500 mg/kg/day) co-administration (G and H). A and E: IgM deposition; B and F: $TCR\alpha\beta^+$ T lymphocytes; C and G: B cells (IgM^+) regions in the recipient spleen; D and H: $TCR\alpha\beta^-$ T cell regions in the spleen.

TABLE 3. Effect of uridine on the ability of leflunomide to control allograft (Balb/c) rejection in C3H mice[a]

Treatment	Allograft survival (d)	Mean±SE
None	8, 9, 9, 10, 10	9.2±0.8
Uridine	8, 9, 9, 10, 11	9.4±1.1
Lef (30)	21, 27, 29, 30, 31	31.0±1.8
Lef (30)+Uridine	19, 19, 20, 30, 31	23.8±2.7

[a] Uridine was administered i.p. at 1000 mg/kg/day (500 mg/kg/dose given twice a day), whereas leflunomide was administered orally at 30 mg/kg daily from the day of the transplant until rejection. Data are presented as mean±SE. On days 5, 10, 12, and 18 after the transplant, 4 other recipients in this group, with functioning xenografts, died of uridine toxicity. This observation indicated that 1000 mg/kg/day is the maximum tolerable dose of uridine. The rest of the C3H recipients were killed on the day of graft rejection.

creased to 35 mg/kg/day, all the Lewis recipients died of, or were killed because of, leflunomide toxicity before the end of the experiment (75 days). The mean survival of the Lewis recipient was 36.0±14.0 days; however, at the time of death or sacrifice, all xenografted hearts were beating (Table 4).

We next tested the effect of uridine coadministration on the ability of leflunomide to prevent acute rejection in this transplantation model. Uridine 500 mg/kg/day coadministration completely antagonized the immunosuppressive activity of 15 mg/kg/day leflunomide, and the xenografts were rejected in 8.4±0.2 days. In contrast, co-administration of uridine 500 mg/kg/day and 35 mg/kg/day leflunomide resulted in long-term xenograft survival for up to 75 days in 3 of 5 recipients; the remaining 2 recipients were killed because of leflunomide toxicity with beating xenografts (Table 4).

As previously reported, pathological analysis of the xenografts on day 50 after the transplant revealed significant vascular injury, indicative of chronic rejection, when leflunomide was used at a dose of 15 mg/kg/day (*26, 27*). Xenografts removed after 10 days of leflunomide monotherapy (15 mg/kg/day) revealed minimal signs of rejection (Fig. 5A, B). A pathological examination of the grafts from rats treated with leflunomide plus uridine (average survival of 8.4 days) revealed severe acute rejection characterized by intense IgM deposition, arterial necrosis, thrombosis, and myocyte coagulating necrosis with a mild to moderate infiltrate comprising neutrophils and macrophages (Fig. 5E, F). At the time of sacrifice of Lewis rats treated with 35 mg/kg/day leflunomide (mean of 36.0±14.0 days after the transplant), the xenografts seemed histologically normal, with no signs of inflammatory cell infiltration or IgM deposition (Fig. 5C, D). Hearts from Lewis recipients treated with leflunomide (35 mg/kg/day) and uridine revealed mild mononuclear intracellular infiltration and IgM deposition, with minimal myocyte necrosis in 4 of 5 grafts (Fig. 5G, H). One graft had severe mononuclear cell infiltration with arterial intimal thickening, a feature characteristic of chronic rejection (data not shown).

We have previously reported that graft rejection in this concordant xenotransplantation model is accompanied by an increase in the titers of xenoreactive IgM but minimal increases in IgG (Fig. 3B) (*17*). We here confirm that leflunomide significantly inhibited the increase in xenoreactive IgM titers at a dose of 15 mg/kg/day and completely inhibited the increase in the xenoreactive IgM titers at the 35 mg/kg/day dose. Immunohistochemical analysis confirmed that leflunomide was able to inhibit xenoantibody production in a dose-dependent manner, with significant IgM deposition in the xenografts on day 75 after the transplant in groups treated with 15 mg/kg/day leflunomide (data not shown) (*26*), and minimal IgM deposition in the 35 mg/kg/day group (Fig. 5B and 5D).

In the groups in which uridine was co-administered with leflunomide, only marginally higher titers of circulating xenoreactive IgM were observed at the 15 mg/kg/day dose, and uridine had no detectable effect on the ability of the higher leflunomide dose to inhibit xenoantibody production. Immunohistochemical analysis indicated minimal IgM deposition in the xenografts from the leflunomide (15 mg/kg/day) monotherapy group on day 10 after the transplant (Fig. 5B), and dense IgM deposition in the xenograft at the time of rejection

TABLE 4. Effect of uridine on the ability of leflunomide to control xenograft (hamster hearts) rejection in Lewis rats[a]

Treatment	Xenograft survival (d)	Mean (d)	Histological scores
None	3, 4, 4, 4, 4, 4, 4, 4, 4	3.9±0.3	4 (AR; X9)
Lef (5)	6, 6, 7, 7, 7, 7, 7, 8, 14	7.7±2.5	4 (AR; X9)
Lef (5)+Uridine	ND		ND
Lef (15)	48, 57, 57, 59, 60, 63, 75, >75[b] >75	>63.2±3.2	4 (CR; X9)
Lef (15)+Uridine	8, 8, 8, 9, 9	8.3±0.25	3B, 4, 4, 4 (AR)
Lef (35)	>24, >30, >31, >35, >60	>35.8±6.3	0, 0, 0, 0, 1A (AR)
Lef (35)+Uridine	>34, >34, >75, >75, >75	>58.3±10	1B, 1B, 1B, 2, 3B

[a] Uridine was administered i.p. at 500 mg/kg (once a day) whereas leflunomide was administered orally at 5, 15, or 35 mg/kg daily for up to 75 days. Survival of Lef-treated Lewis rats or grafts were calculated from the day of the transplant and presented as mean±SE. Xenografts were harvested on the day the rats were killed or at the end of experiment (day 75 after the transplant). Histological scores were determined as described under Materials and Methods.

[b] > indicates that the Lewis recipients died or were killed with beating grafts on day 75 after the transplant. AR, acute rejection; CR, chronic vascular rejection.

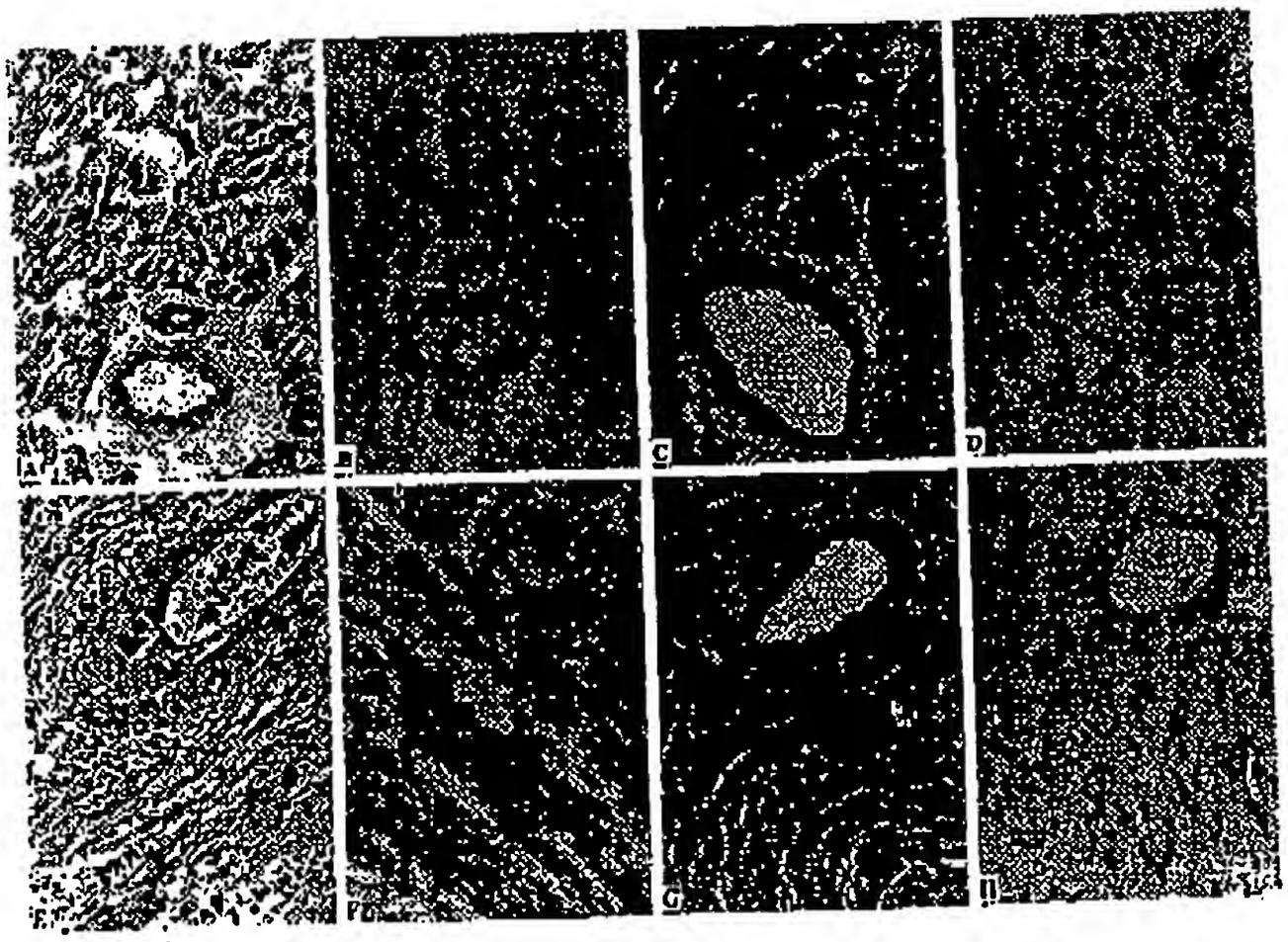

FIGURE 5. The ability of leflunomide (15 mg/kg/day (A, B, E, F) and 35 mg/kg/day) (C, D, G, H) to inhibit xenograft rejection was significantly reversed by uridine co-administration (E–H). Xenografts were harvested on day 10 after transplant (A and B), rejection (D and F), day of sacrifice (C and D), or day 75 after transplant (G and H). A, C, E, and G: HE staining; B, D, F and H: IgM immunostaining.

(day 8–10 after the transplant) in the group treated with 15 mg/kg/day of leflunomide plus uridine (Fig. 5F). There was also marginally more IgM deposition in the xenografts on day 75 in the groups treated with 35 mg/kg/day of leflunomide plus uridine (Fig. 5H), compared with the leflunomide monotherapy group (Fig. 5D). These results indicate that uridine can reduce the ability of leflunomide to control xenoreactive IgM production in the hamster-to-Lewis concordant xenotransplantation model.

Effect of uridine on the toxic side-effects of leflunomide in Lewis rats. Lewis rats with either an allograft or a xenograft, and treated with leflunomide at a dose of 35 mg/kg/day, survived for a mean of 30.5 days (Fig. 6A), with only one of 10 rats surviving <50 days. When the same dose of leflunomide was administered with uridine (500 mg/kg/day), the mean survival of Lewis rats was significantly enhanced, and 7 of 10 Lewis rats were alive after 50 days (Fig. 6A). These observations suggest that this dose of uridine could antagonize the toxic side effects of high-dose leflunomide. Lewis recipients treated with 15 mg/kg/day of leflunomide alone, or in combination with uridine, exhibited minimal signs of toxicity.

Typical signs of leflunomide toxicity in Lewis rats are anemia and diarrhea. We measured the percent of PCV every 14 days after the transplant in the Lewis rats receiving either allografts or xenografts. Treatment with 35 mg/kg/day of leflunomide resulted in a rapid decline in the percentage of PCV (Fig. 6B). In contrast, in the groups receiving uridine and 35 mg/kg/day of leflunomide, the decline in percent of PCV was delayed (Fig. 6B). In the Lewis recipients treated with 15 mg/kg/day leflunomide alone, or combination with uridine, there was no significant drop in the percentage of PCV for the duration of the experiment.

At the end of the experiment (either natural death or being killed), Lewis rats were subjected to a complete autopsy. Histological signs of leflunomide toxicity (35 mg/kg/day) were observed primarily in the small bowel and liver (Fig. 7A, B). Epithelial abnormalities were observed in the intestinal mucosa of the small bowel of rats treated with high-dose leflunomide (35 mg/kg/day). In particular, the villi were short and wide and mature intestinal epithelial cells, including goblet cells, brush border absorption epithelium, and Paneth's cells were partially or completely substituted by immature low columnar cells, with or without dysplasia (Fig. 7A). These epithelial abnormalities could be caused by atrophy, dedifferentiation, or inhibition of regeneration of the intestinal mucosa by high-dose leflunomide. Uridine significantly pre-

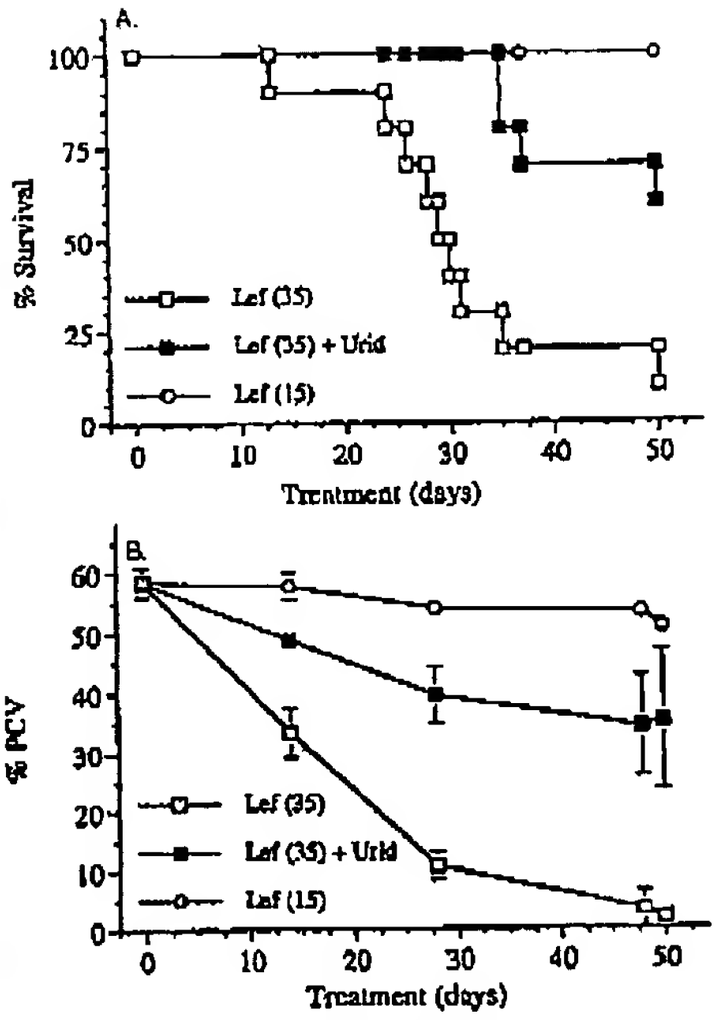

FIGURE 6. (A) Survival of Lewis recipients after treatment with leflunomide (Lef; 15 or 35 mg/kg/day) alone or in combination with uridine (Urid; 500 mg/kg/day). (B) The hematocrits in Lewis recipients after treatment with leflunomide (Lef; 15 or 35 mg/kg/day) alone or in combination with uridine (Urid; 500 mg/kg/day). Data are presented as mean PCV of 10 rats±SE.

vented changes in the small bowel in the three surviving Lewis rats treated with leflunomide and uridine for ≥75 days (Fig. 7C).

Toxicity in the liver was characterized by fatty degeneration, atrophy, and necrosis of the hepatocytes in the central lobular regions (Fig. 7B). These changes were completely abrogated with the co-administration of uridine, and the findings of the histological analysis of the liver seemed normal (Fig. 7D).

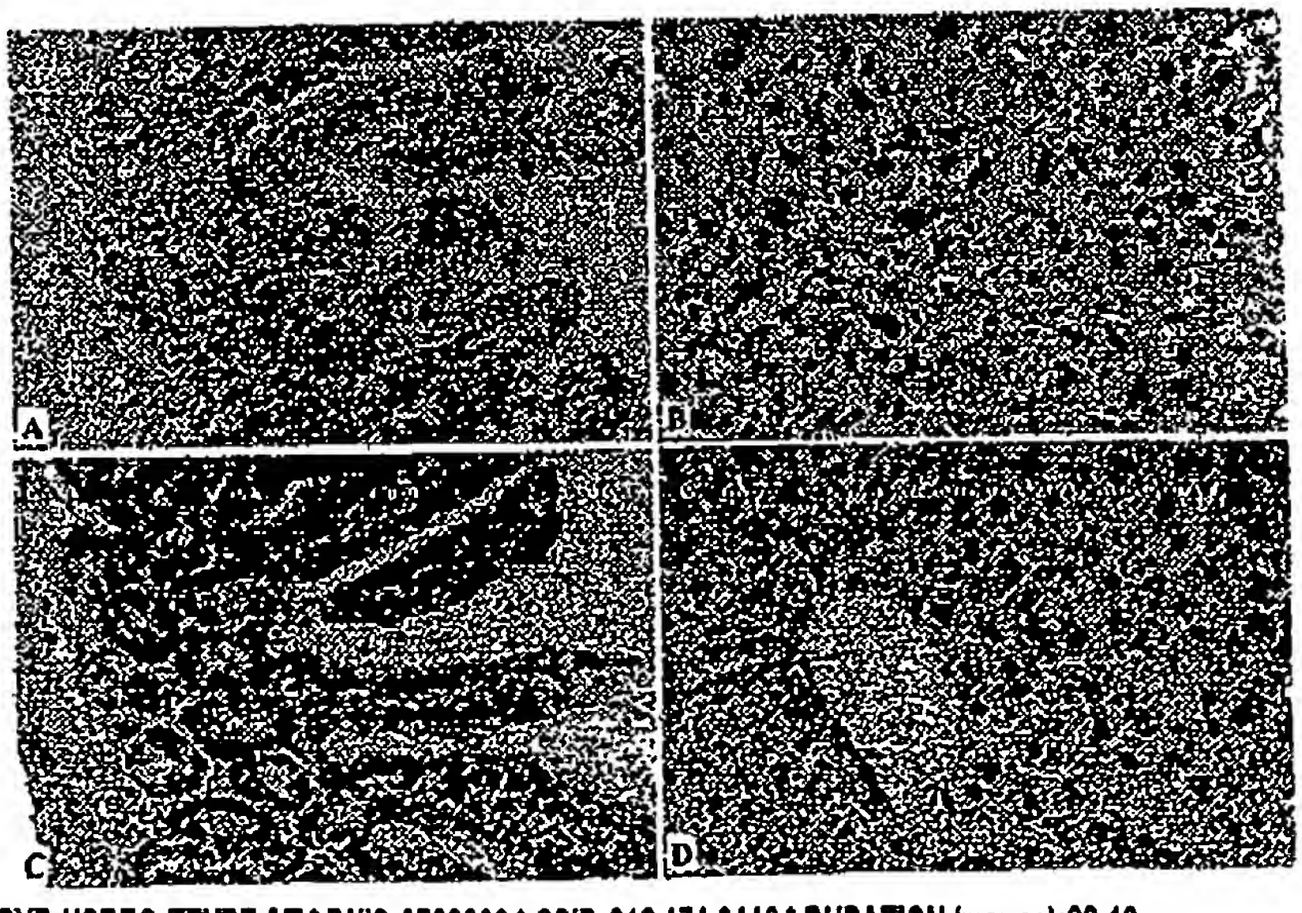

FIGURE 7. Histological examination of the effects of leflunomide (35 mg/kg/day) monotherapy (A, B), or in combination with uridine (C, D) in the small bowel (A, C), and liver (B, D). Liver and small bowel were stained with hematoxylin and eosin.

DISCUSSION

When relating the *in vitro* activity of a drug to its *in vivo* activity, it is usually necessary to assess the drug levels in the blood and tissue, at doses known to modify the function of the target organ system. With leflunomide, two *in vitro* activities are known to exist at two distinct concentrations: inhibition of *de novo* pyrimidine synthesis and selective inhibition of tyrosine kinases. Either of these activities might account for the immunomodulatory activity of leflunomide. To individually assess these activities *in vivo* requires that at least one of the activities be controlled. Uridine can be used as an antidote for inhibition of *de novo* pyrimidine synthesis; thus, we first conducted a pharmacokinetic analysis of serum A77 1726 after the administration of leflunomide, and of serum uridine and tissue nucleotide triphosphate levels after the administration of uridine.

After a single oral administration of leflunomide (5, 15, and 35 mg/kg), we observed a linear dose-dependent relationship between the dose administered and the concentration of A77 1726 in the serum. Consistent with previous studies, the increase in A77 1726 levels in the serum was relatively slow (Ka = 1.97 to 0.484 mL · min/kg) and peak levels were reached in 6–8 hr, irrespective of the dose (*28, 29*). After the administration of a single, i.p. dose 500 mg/kg of uridine in Lewis rats, increased levels of serum uridine were detected almost immediately and the peak concentration of uridine in the serum, 384.1±53.5 μM/L, was observed within 15 min (*28, 29*). However, uridine plasma levels returned to normal by 4 hr, indicating that the pharmacokinetics of uridine are significantly different from that of A77 1726. The levels of UMP and CTP in the spleen, lymph nodes, and liver of Lewis rats were elevated within 1 hr after the administration of 500 mg/kg uridine. In contrast to rapid elimination of serum uridine, the levels of UTP and CTP in the spleen, liver, and lymph nodes remained elevated for 6–12 hr. Our data further suggest a hierarchy in the duration of elevated pyrimidine nucleotides in the spleen>liver>lymph nodes, perhaps reflecting the ability of different tissues or cells to salvage uridine and the availability of serum uridine (*30, 31*). A

critical role of the spleen in the development of cellular and antibody responses is suggested by the central position of the spleen in blood circulation and the large numbers of lymphocytes migrating. Thus, it is noteworthy that the pharmacokinetic findings of UTP levels after uridine administration in the spleen is most similar to that of serum A77 1726 after administration of leflunomide.

We next examined the effects of uridine on the immunosuppressive activity and toxicity of leflunomide in Lewis rats receiving with brown Norway or Golden Syrian hearts. On the basis of three criteria: survival, histological examination of the allograft, and titers of alloreactive antibodies, we conclude that the ability of leflunomide to control allograft rejection seems to be only minimally affected by uridine coadministration. It is interesting that the lymphoid areas in the spleen that were significantly reduced in leflunomide-treated rats receiving allograft hearts were significantly reversed by uridine co-administration. We, therefore, speculate that the immunosuppressive effects of leflunomide in this allotransplantation model are independent of the DHO-DHase-dependent antiproliferative effects of leflunomide. In a second allograft model (Balb/c into C3H), uridine also had modest effects on the immunosuppressive activity of leflunomide. These data are consistent with the conclusion that the mechanism by which leflunomide controls alloreactivity is largely independent of inhibition of pyrimidine synthesis *in vivo*.

The modest effect of uridine in this allograft model contrasts with our *in vitro* data that indicate that uridine can completely antagonize the antiproliferative activity of the active metabolite of leflunomide, A77 1726, when it is used at concentrations that are ≤25 μM (9). However, we had noted that uridine only partially reversed the antiproliferative effects of A77 1726 when the concentrations were ≥50 μM, and had no effect of the ability of A77 1726 to inhibit T cell cytotoxic activity (9). Because A77 1726, at IC_{50} of ≥50 μM, inhibits tyrosine phosphorylation in lymphocytes, we had hypothesized that the immunosuppressive activity at ≥50 μM A77 1726, and in the presence of uridine, results from inhibition of tyrosine phosphorylation (6, 9, 32).

Single-dose pharmacokinetic studies of rats treated with leflunomide at 5, 15, and 35 mg/kg/day indicated that peak concentrations of A77 1726 in the sera were 77.5, 266.1 and 478.8 μM, respectively, whereas the 24 trough concentrations are 4.9, 6.3, and 125 μM, respectively. Additionally, single-dose pharmacokinetic studies of mice treated with leflunomide at 35 mg/kg/day indicates that the peak concentration of A77 1726 in the sera is 518.2 μM, whereas the 24 hr trough concentration is 227.5 μM (data not shown). Therefore, the inability of uridine to counter the effects of leflunomide in this allograft model is consistent with the *in vitro* observations that uridine cannot reverse the immunosuppressive activities of higher doses of A77 1726 *in vitro* (9). We, thus, conclude that the primary mode of immunosuppression by leflunomide in this allograft model may be related to the inhibition of tyrosine phosphorylation and that the inhibition of *de novo* pyrimidine synthesis is of secondary importance.

In contrast to the modest effect of uridine in the allograft model, the ability of uridine to antagonize the immunosuppressive activity of 15 mg/kg/day of leflunomide in the xenograft model is very convincing. In the leflunomide monotherapy groups (15 mg/kg/day), the xenografts survive for a mean of >63 days. There was minimal IgM deposition in the xenograft on day 10 but significant IgM at the time of rejection or on day 75 after the transplant. In the combination therapy group, the xenografts were rejected in 8–10 days, and rejection was associated with extensive deposition of IgM in the xenograft. At the higher dose of leflunomide (35 mg/kg/day), the effect of uridine was more modest and the xenograft hearts were still beating at the time the rats were killed (day 34 or day 75 after the transplant). A histological examination of the xenografts from the combination treatment group (leflunomide [35 mg/kg/day] plus uridine) revealed increased signs of inflammation, chronic rejection, and IgM deposition, compared with the 35 mg/kg/day leflunomide monotherapy group. These observations suggest that inhibition of *de novo* pyrimidine synthesis is an important part of immunosuppressive therapy in the xenotransplantation model. It further suggests that insufficient uridine is not the explanation for our observations in the allotransplantation model.

The contrasting effect of uridine on the immunosuppressive activity of leflunomide may result from different mechanisms of rejection in the allograft versus the xenograft model. Acute xenograft rejection is dependent on the rapid production of xenoreactive IgM; in contrast, acute allograft rejection is a T-cell dependent process (*17, 33*). Therefore, it is possible that the control of B cell function in xenograft rejection by leflunomide depends more on the inhibition of pyrimidine synthesis, whereas the control of T cells by leflunomide may be more dependent on the inhibition of tyrosine phosphorylation. *In vitro* observations suggest that B cells may be more susceptible to the effects of inhibition of *de novo* pyrimidine synthesis than T cells (*9, 10, 34–36*).

A second goal of these studies was to investigate whether uridine could be used to control leflunomide-induced toxicity. The most consistent symptoms in Lewis rats treated with 35 mg/kg/day of leflunomide were severe anemia and diarrhea resulting in weight loss, dehydration and, ultimately, death. Uridine was able to significantly reduce the anemia and prolong the survival of the Lewis rats. Autopsies revealed liver necrosis and pathological changes in the small bowel in rats treated with 35 mg/kg/day leflunomide. Most of these changes were significantly reversed by uridine co-administration, suggesting that the toxicity of the liver and small bowel, and the inhibition of hematopoiesis, is largely caused by inhibition of pyrimidine synthesis.

In summary, we report that uridine had minor effects on the immunosuppressive activity of leflunomide in the allograft model, and a more significant effect in the xenograft model. Thus, it seems that the mechanism of immunosuppression by leflunomide *in vivo* is complex and may be affected by at least the following four factors: the type and vigor of the immune response; the availability of uridine for salvage by proliferating lymphocytes; the species-specific efficacy by which leflunomide inhibits the activity of dihydroorotate dehydrogenase, and the levels of A77 1726 *in vivo*.

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(54) Title: METHODS AND COMPOSITIONS FOR REDUCING TOXICITY ASSOCIATED WITH LEFLUNOMIDE TREATMENT

(57) Abstract: The invention relates to methods and compositions useful in alleviating or reducing toxicity associated with leflunomide administration without reducing its bioactivity, e.g., without reducing its immunosuppressive activity, that is, utilizing a bioavailable pyrimidine compound to ameliorate the toxic effects caused by leflunomide compounds.

METHODS AND COMPOSITIONS FOR REDUCING TOXICITY ASSOCIATED WITH LEFLUNOMIDE TREATMENT

Leflunomide is an isoxazole derivative which has shown therapeutic potential in a diverse array of disease processes and conditions, e.g., as an antiinflammatory agent, an immunosuppressive agent, an anticancer agent and an antiviral agent.

Leflunomide is currently approved in the United States for use in the treatment of rheumatoid arthritis to reduce joint inflammation. It is marketed under the trademark ARAVA®.

U.S. Patent Nos. 5,624,946 and 5,688,824, incorporated herein by reference in their entirety, report that leflunomide has been used experimentally as an immunosuppressive agent in the treatment and prevention of chronic rejection in xenograft and allograft transplant recipients, both alone and in combination with other immunosuppressive agents.

In addition to data suggesting its value in treating, preventing and reversing acute and chronic rejection, U.S. Patent Application U.S. 2003/0114597, incorporated herein by reference in its entirety, reports that leflunomide has been shown to inhibit viruses of the Herpesviridae family *in vitro*.

U.S. Patent No. 4,965,276 describes the use of leflunomide to treat chronic graft versus host disease and other autoimmune diseases such as systemic lupus erythematosus (SLE). Leflunomide has also been shown to exhibit antineoplastic activity against certain tumors (Xu X et al., *Biochem. Pharmacol.* 1999; 58:1405) and may act by inhibiting tumor neoangiogenesis (Waldman WJ et al., *Transplantation* 2001; 72:1578).

Despite the reported therapeutic benefits of leflunomide in the treatment and prevention of these disease processes, it has also been noted that administration of leflunomide may produce dose-limiting toxicity. Toxicity associated with high doses of leflunomide include anemia, diarrhea, and pathological changes of the small intestine and liver. In a study of the anti-cancer effects of leflunomide (inhibition of the oncogene product PDGF and PDGFr) observable beneficial effects were reported but the doses required for these effects produced unacceptable incidence of side effects, including severe weight loss, anorexia and anemia. (Ko, Yoo-Joung, et al. *Clinical Cancer Research*, 2001;7: 800-805)

Recently, it has been suggested that uridine therapy reduces the toxicity of leflunomide without significantly impairing the control of allograft rejection afforded by leflunomide. The utilization of exogenous uridine occurs through the pyrimidine salvage

pathway in the face of the leflunomide's blockade of the *de novo* pathway. Notwithstanding the potential benefits of administration of exogenous uridine, therapeutic use of uridine is complicated by its poor bioavailability (about 8% - 10%), requiring high dose administration for effective therapy. Moreover, high doses of uridine may cause gastrointestinal complications, including diarrhea, which are poorly tolerated in transplant patients dependent on intestinal function for therapeutic drug administration and which may exacerbate the diarrhea already caused by the leflunomide.

The present invention relates to the surprising discovery that the use of orotic acid alleviates the toxicity typically observed with leflunomide administration. Orotic acid (also known as vitamin B_{13}), an intermediate in the uridine synthetic pathway, appears to eliminate the pyrimidine deficiency caused by the malononitrilamides, metabolites (analogues of the active metabolite) of leflunomide, while avoiding the problems associated with uridine administration.

Accordingly, the invention provides methods and compositions useful in alleviating or reducing toxicity associated with leflunomide administration without reducing its bioactivity, e.g., without reducing its immunosuppressive activity. The present invention uses a bioavailable pyrimidine compound to ameloriate the toxic effects (e.g., anemia, diarrhea, heptotoxicity) caused by leflunomide compounds. As a result, high doses of leflunomide compounds can be administered with minimal danger of toxicity, all the while maintaining the therapeutic efficacy of the leflunomide compound. Co-administration of a leflunomide compound with orally bioavailable pyrimidines, such as orotic acid, provides for treatment opportunities using leflunomide compounds previously believed to be toxic, e.g., the present invention provides methods of reducing the toxicity of A77 1726 (a metabolite of leflunomide) analogs (described hereinbelow) by co-administering a leflunomide compound and, e.g., orotic acid. In addition to orotic acid, it is contemplated that additional analogs and metabolites of orotic acid or other bioavailable pyrimidine compounds may be suitable.

In one aspect, the invention provides pharmaceutical compositions particularly for oral administration. Such pharmaceutical compositions suitably include a leflunomide compound, a bioavailable, especially an orally bioavailable, pyrimidine compound or a salt thereof, and a pharmaceutically acceptable carrier.

In another aspect, the invention provides a method of extending the dosage range of a leflunomide compound. The method involves co-administering to a subject, e.g., a mammal, an effective dose of a leflunomide compound and an orally bioavailable

pyrimidine compound or salt thereof, e.g., orotic acid. Thus, the invention provides a method of administering high doses of a leflunomide compound without developing, i.e., reducing, toxicity resulting from leflunomide administration, which method comprises administering to a mammal, e.g., a human, treated with a leflunomide compound an effective amount of a bioavailable pyrimidine compound.

The invention further provides methods of prevention or treatment of certain disease states or processes that are suitably treated with a leflunomide compound. Such disease states or conditions include transplant rejection.

The invention will now be described in detail, those skilled in the art will appreciate that such a description of the invention is meant to be exemplary only and should not be viewed as limitative of the full scope thereof.

The following definitions used in the art may be useful in aiding the skilled practitioner in understanding the invention.

"Ameliorating" means observably reducing, alleviating, inhibiting or diminishing any undesirable effect or symptom of a condition or process associated with a disease state or any undesirable effect of a treatment of a disease state. For example, "amelioration of the effects of pyrimidine biosynthesis inhibition" may refer to any observable reduction in side effects caused by pyrimidine biosynthesis inhibition. Suitably, at least a 50% reduction in symptoms or side effects may be observed.

The term "co-administration" includes administration of two or more agents in a single unitary dosage form, administration of agents concurrently, and administration of agents sequentially, as long as they are given in a manner sufficient to allow both agents to achieve effective concentrations in the body. The agents may be in an admixture, as, for example, in a single tablet, or simply given concurrently. The agents may also be administered by different routes, e.g., one agent may be administered intravenously while the second agent is administered orally. In sequential administration, one agent may directly follow administration of the other or the agents may be administered episodically, i.e., one can be given at one time followed by the other at a later time.

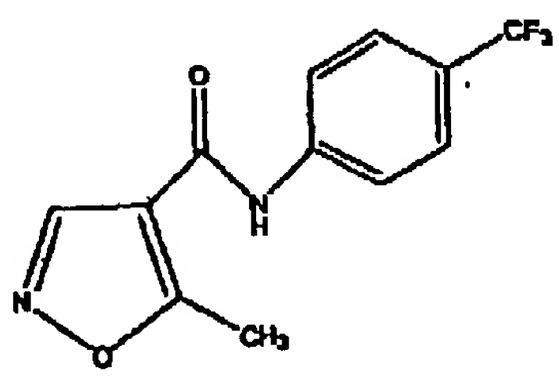
An "effective amount" of a compound, as used herein, means that amount of the compound or composition administered to a subject which is effective to produce its intended function, e.g., in one embodiment of the invention, prevention of transplant rejection. Thus, a "therapeutically effective amount" is an amount effective to produce therapeutic results. A "toxicity-reducing effective amount" is an amount effective to reduce toxicity. Typically, administration of effective amounts to a subject results in

observable amelioration of undesirable effects or symptoms of the condition or disease process which the subject is being treated.

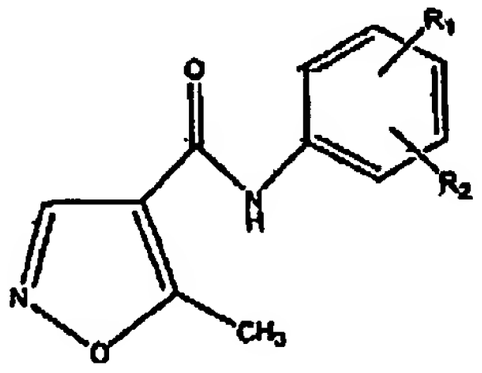
"Extending a dosage range" refers to providing a means by which greater doses of an agent may be administered to a subject to increase therapeutic effectiveness. Typically, extending a dosage range is useful, e.g., when efficacy of an agent is dose dependent but increased doses of the agent also leads to dose dependent toxicity. Alternatively, the term "extending a dosage range" may refer to administering agents believed to be toxic at any dosage.

A "leflunomide compound" refers generally to leflunomide, its analogs, its metabolites and analogs thereof.

Leflunomide is an isoxazole derivative with a chemical name of N-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxamide having the following chemical formula (I):



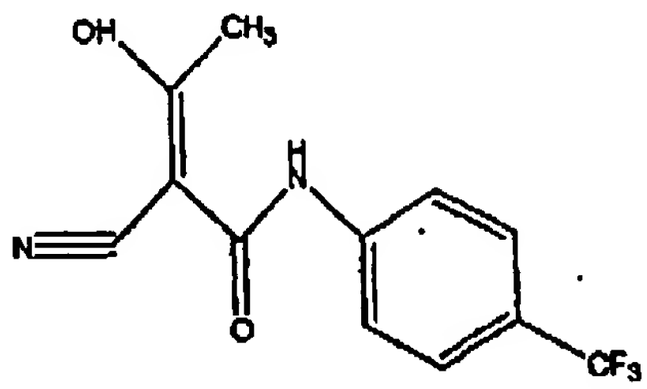
Analogs of leflunomide which may be useful in the practice of the methods of the invention may be represented by formula (II):



(I) (II)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, -H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, $-NH-CO-CH_2Cl$ and $NH-CO-CH_2Br$. (See, e.g., U.S. Patent Nos. 4,087,535; 6,133,301; and 6,727,272)

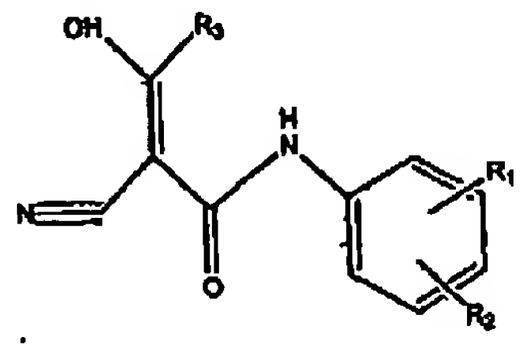
Leflunomide's active metabolite is referred to as "A77 1726" (2 cyano-3hydroxy-N-(4-trifluromethylphenyl)-buteneamide). After administration, leflunomide is rapidly converted to its active open-ring form, A77 1726, and is shown herein as formula (III):



(III)

This compound, a member of the malononitrilamide class of compounds, appears to account for leflunomide's activity and toxicity. Although its mechanism of action is not completely understood and wishing not be bound of any particular theory, A77 1726 is believed to exhibit at least two biochemical activities *in vivo*: inhibition of dihydroorotic acid dehydrogenase (DHODH) in the *de novo* synthesis of pyrimidine nucleotide triphosphates; and inhibition of selected tyrosine kinases involved in T-cell, B-cell, vascular smooth muscle cell, endothelial cell, fibroblast and tumor cell signaling cascades. A77 1726 also has been reported to block NFkB and AP-1 activation in peripheral blood lymphocytes *in vitro*. Additional mechanisms remain to be discovered.

Suitable malononitrilamide compounds which are analogs of A77 1726 may be useful in the practice of the methods of the invention and may be represented by formula (IV):



(IV)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, -NH-CO-CH_2Cl and NH-CO-CH_2Br and wherein R_3 is selected from the group consisting of C_{1-5} alkyl, C_{2-5} alkenyl, C_{2-5} alkynyl, and C_{3-6} cycloalkyl. Compounds of formula (IV) include FK7778 and FK779 wherein R_1 is -H, R_2

is $-CF_3$ and R_3 is butynyl (i.e., 2-cyano- 3-hydroxy-N-[4-(lufluaromethyl) phenyl]-2-hepten-6-ynoic acid amide) and R_1 is -H, R_2 is cyano and R_3 is cyclopropyl (i.e., 2-cyano-3-hydroxy-3-cyclopropyl-N-(4-cyanophenyl)-propenic acid amide), respectively.

In some embodiments of the methods of the invention, the leflunomide compound is administered as a prodrug to subjects and subsequently converted *in vivo* to its active malononitrilamide compound, defined above. It is contemplated, however, that the malononitrilamide compound may also be directly administered, and the term "leflunomide compound", as defined above, also refers to malononitrilamide compounds. It is to be understood that discussion herein regarding leflunomide compound administration is meant to be inclusive of malononitrilamide compound administration, as appropriate.

Leflunomide and its analogs can be prepared by known methods such as those described in U.S. Patent No. 6,723,855; U.S. Patent No. 6,727,272; U.S. Patent No. 6,133,301; U.S. Patent No. 5,905,090; U.S. Patent No. 4,087,535; U.S. Patent No. 4,351,841; and U.S. Patent No. 4,965,276, all of which are incorporated herein by reference in their entireties. Leflunomide is also commercially available from chemical suppliers, such as SynQuest Corp. (Chicago, Illinois).

As used herein, "bioavailable" in reference to a pyrimidine compound is one that is at least about 20% bioavailable after administration. "Orally bioavailable" in reference to a pyrimidine compound is a compound that is at least about 20% bioavailable after oral administration.

The phrase "pharmaceutically acceptable carrier," as used herein, means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject chemical from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and

polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations. Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

As used herein, "preventing," "reducing risk" or "reduced risk" as it applies to a particular condition or disease process, refers to observable results which tend to demonstrate that a particular treatment or treatment regimen has resulted in a significant decrease in incidence of the condition or disease process in a treated population, as compared to an untreated or control population. Suitably, risk is reduced, or a condition is prevented, if at least 50% of the treated population are not afflicted.

As used herein, a "pyrimidine compound" refers to a compound that is bioavailable, especially orally bioavailable, and useful either directly or as intermediates in pathways for supplying pyrimidine nucleotides. A suitable pyrimidine compound is, e.g., orotic acid. Other suitable pyrimidine compounds include orotic acid salts, triacetyl uridine and salts thereof, cytidine, acylated cytidine and salts thereof.

It is to be understood that the phrase "a salt thereof," when used herein to refer to pharmaceutical compositions, means physiologically compatible salts which are pharmaceutically acceptable. Examples of suitable salts are alkali metal (e.g., sodium), alkaline earth metal (e.g., calcium, magnesium) and ammonium salts, including those of physiologically tolerated organic ammonium bases.

As used herein, the term "treating" means observably reducing any undesirable effect or symptom of a condition or process associated with a disease state or any undesirable effect of a treatment of a disease state. Suitably, at least a 50% reduction in symptoms or side effects may be observed in a treated subject.

It also is specifically understood that any numerical value recited herein includes all values from the lower value to the upper value, i.e., all possible combinations of numerical values between the lowest value and the highest value enumerated are to be considered to be expressly stated in this application. For example, if a concentration range is stated as 1% to 50%, it is intended that values such as 2% to 40%, 10% to 30%, or 1%

to 3%, etc., are expressly enumerated in this specification. These are only examples of what is specifically intended.

In one embodiment, the present invention provides an effective method for reducing the risk of toxicity of leflunomide compounds used for the treatment of transplant rejection. Particularly, the present invention relates to therapeutic methods for ameliorating the risk of toxic side effects of a leflunomide compound, and thus permitting extending dosing of such compounds. The present invention provides treatment of a patient suffering from the toxic side effects of a leflunomide compound with an orally bioavailable pyrimidine compound based on a novel treatment protocol. The pyrimidine compound is suitably orotic acid, a salt thereof (e.g., sodium orotate), or a triacetyluridine. The pyrimidine compound is provided to the patient to significantly reduce the toxic effects of a leflunomide compound, e.g., anemia and diarrhea resulting in reduced hematocrit and weight loss. These attributes are achieved through specific properties of the pyrimidine compounds and the novel treatment protocol as described herein.

A suitable pyrimidine compound is orotic acid. Orotic acid is found in small concentrations in the blood of healthy individuals. Elevated levels appear to be free of any appreciable complications in humans and animals. Several conditions are known, however, in which orotic acid levels in the blood are elevated, e.g., in urea synthesis defects, in individual Hereditary Oroticaciduria treated with uridine for years, and in patients receiving allopurinol, without recognized specific damage. In addition, it is recognized that blood levels of orotic acid are elevated several fold in renal failure without specifically recognized toxicity.

Orotic acid may be prepared by condensation of urea with the monoethyl ester of oxalacetic acid in methanol. Other preparation methods, including those utilizing biotechnological methods known in the art, are also suitable. Orotic acid may be administered in its free acid form, or may be administered as a pharmaceutically acceptable salt. Examples of suitable salts are alkali metal (e.g., sodium orotate), alkaline earth metal (e.g., magnesium orotate or calcium orotate) and ammonium salts, including those of physiologically tolerated organic ammonium bases. Orotic acid is also commercially available from chemical suppliers, such as Aldrich (Milwaukee, Wisconsin).

Also included among the bioavailable pyrimidine compounds of the invention are those comprising certain known acyl derivatives of uridine, i.e., acylated uridines, e.g., 2', 3', 5'-tri-O-acetyl uridine (or triacetyluridine (TAU)), 2', 3', 5'-tri-O-propionyl uridine, or 2', 3', 5'-tri-O-butyryl uridine. TAU, for example, is orally bioavailable. TAU and other

acyl derivatives of uridine can be made by methods known in the art (see, e.g., U.S. Patent No. 6,316,426; U.S. Published Patent Application 2002/0035086 and references cited therein, all of which are incorporated herein by reference); TAU is also commercially available through SP-Chemicals, Ludwigshofen, DK.

The pyrimidine compounds of the invention also include cytidine and certain acyl derivatives of cytidine, i.e., acylated cytidines, e.g., 2', 3', 5'-tri-O-acetyl cytidine (or triacetylcytidine. or TAC), 2', 3', 5'-tri-O-propionyl cytidine, or 2', 3', 5'-tri-C-butyryl cytidine. TAC and other acyl derivatives of cytidine can be made by methods known in the art (see, e.g., U.S. Published Patent Application 2002/0035086 and references cited therein, all of which are incorporated herein by reference).

Suitably, the pyrimidine compound may be administered in an amount that is approximately that which is needed to provide the daily pyrimidine synthesis requirements minus what is provided through the salvage pathway. The total pyrimidine synthesis in adult humans is estimated to be from about 4 mmol/day to about 12 mmol/day, or about 450 to about 700 mg of uridine per day. (Bono VH, Weissman SM, Frei E. The effect of 6-azauridine administration on de novo pyrimidine production in chronic myelogenous leukemia. *J Clin Invest* 1964; 43:1486; Smith LH Jr. Pyrimidine Metabolism in Man. *New Engl J of Med* 1973; 288:764-772.) For orotic acid, this would amount to approximately 1000 mg per day. It is not believed to be necessary, however, to provide the entire daily supply of pyrimidine since the salvage pathway provides some of the total. It is believed that the bioavailability of orotic acid is approximately 50%. Therefore, for oral administration in an adult, an effective amount of orotic acid would be about 500 mg to about 2,000 mg per day. A similar dosing is contemplated for TAU.

For patients being treated with a leflunomide compound, the targeted blood level of active metabolite (A77 1726) is suitably between about 50 μg/mL and about 100 μg/mL. The maintenance dose may be adjusted by one of ordinary skill in the art to attain the desired blood level range of active metabolite. If pyrimidine deficiency is prevented with co-administration of a pyrimidine compound, the targeted blood level of active metabolite may be substantially higher, e.g. about 200 μg/mL or about 600 μM.

Mammalian transplant recipients, such as kidney recipients and bone marrow recipients, may be suitably treated in accordance with the present invention. Typically, a human transplant recipient is administered a leflunomide compound at a dose of about 100 mg per day for five days, and then 40 mg per day thereafter as a maintenance dose. The co-administration of the leflunomide compound and a pyrimidine compound will extend

the therapeutic dose of the leflunomide compound to more than 200 mg/patient/day. This method will prevent the development of or reduce the risk of toxicity (e.g., anemia, diarrhea, hepatotoxicity) and will result in achieving concentrations of the leflunomide compounds that can suppress rejection. It is expected that the use of this methodology will allow up to 10-fold or higher increase in dosage level of leflunomide compounds with minimal danger of developing toxicity to the patient. In other words, the present invention provides a method of administering a toxic dose of a leflunomide compound by administering an effective amount of a pyrimidine compound. By "toxic dose" or "high dose" is meant a dose of the leflunomide compound which when administered to a mammal such as a human often results in the toxic effects, e.g., anemic and diarrhea as well as other pathological changes. In humans, a high dose may be more than 200 mg per day.

In administration of leflunomide compounds, toxicity-reducing effective amounts of the bioavailable pyrimidine compounds are co-administered to subjects with allografts or xenografts, thereby ameloriating the toxic effects of the leflunomide compounds, i.e., weight gain is promoted and hematocrit maintained, with significantly less risk of toxicity than is observed after the same amount of leflunomide compound alone is administered. The risk of toxicity, associated with the administration of high doses of leflunomide compounds, is lowered by co-administering the leflunomide with a pyrimidine compound, especially an orally bioavailable pyrimidine compound. Thus, the combination therapy for use in accordance with the present invention provides an improved therapeutic index relative to leflunomide compounds alone given in conventional protocols. The treatment protocol in accordance with the present invention provides reduced risk of toxicity, (e.g., improved weight gain and hematocrit) i.e., little or no clinical symptoms or signs of toxicity.

The pyrimidine compounds of the present invention given in the illustrated dosing regimen, thus, overcome the toxicities of leflunomide compounds and can be considered beneficial agents for the control and treatment of toxicity associated with treatment with leflunomide compounds. In such combination therapy, the leflunomide compound may be co-administered with the pyrimidine compound concurrently, sequentially, or in a unitary formulation. For efficiency, ease of administration and patient compliance, the latter is especially suitable.

A pharmaceutical composition of a leflunomide compound and a bioavailable pyrimidine compound is suitably formulated in unit dosage form of about 500 mg to about

2000 mg of pyrimidine compound and about 20 mg to about 100 mg of leflunomide compound. Lower doses of pyrimidine compound may be adequate for children or individuals with reduced clearance of pyrimidines, such as individuals with reduced kidney function or other conditions that might reduce pyrimidine elimination.

The dosage form of compositions of the invention is not particularly limited, and any form suitable for oral administration may be used in accordance with standard formulation procedures known in the art. Examples of dosage forms suitable for oral administration include, but are not limited to, solid formulations and aqueous formulations. Solid formulations suitable for oral administration include capsules, tablets, powders or granules, and may include excipients such as lactose, glucose, sucrose or mannitol; a disintegrator such as starch or sodium alginate; a lubricant such as magnesium stearate or talc; a binder such as polyvinyl alcohol, hydroxypropylcellulose or gelatin; a surfactant such as fatty acid ester; and a plasticizer such as glycerine, and the like. Aqueous formulations suitable for oral administration include solutions, emulsions, syrups and suspensions. Such formulations may also include sugars such as sucrose, sorbitol or fructose; glycols such as polyethylene glycol or propylene glycol, oils such as sesame oil, olive oil or soybean oil, antiseptics such as p-hydroxybenzoate, and flavors such as strawberry and peppermint.

While, perhaps, less convenient than an oral formulation, it is also contemplated that the compositions may be formulated for rectal administration in accordance with standard formulations procedures known in the art. Examples of dosage forms suitable for rectal administration include solid suppositories, mucoadhesive suppositories, solutions, suspensions, retention enemas, gels, forms and ointments.

It is further contemplated that a dosage form of the compositions in accordance with the present invention may be formulated for immediate release, delayed release or controlled release. Many controlled release systems are known in the art (see e.g., U.S. Patent 5,529,991). Sustained, controlled or directed release compositions can be formulated, e.g., in liposomes, via laser originated openings or those wherein the active compound is protected with differentially degradable coatings, such as by microencapsulation, multiple coatings, etc.

For example, in diffusional systems, the release rate of drugs is affected by their rate of diffusion through a water-insoluble polymer. There are generally two types of diffusional systems, formulations in which a core of drug is surrounded by polymeric membrane; and matrix devices in which dissolved or dispersed drug is distributed

substantially uniformly and throughout an inert polymeric matrix. In actual practice, many systems that utilize diffusion can also rely to some extent on dissolution to determine the release rate.

Common materials used as the membrane barrier coat, alone or in combination, include but are not limited to, hardened gelatin, methyl and ethyl-cellulose, polyhydroxymethacrylare, polyvinylacetate, and various waxes.

In matrix systems, three major types of material are frequently used in the preparation of the matrix systems which include insoluble plastics, hydrophilic polymers, and fatty compounds. Plastic matrices which have been employed include methyl acrylate-methyl methacrylate, polyvinyl chloride and polyethylene. Hydrophilic polymers include methyl cellulose, hydroxypropylcellulose, hydroxpropyl-ethylcellulose, and its derivatives and sodium carboxy-methylcellulose. Fatty compounds include various waxes such as carnauba wax, and glyceryl tristearate. These matrix systems are prepared by methods well known to those skilled in the art. These methods of preparation generally comprise mixing the drug with the matrix material and compressing the mixture into a suitable pharmaceutical layer. With wax matrices, the drug is generally dispersed in molten wax, which is then congealed, granulated and compressed into cores.

The most common method of microencapsulation is coacervation, which involves addition of a hydrophilic substance to a colloidal dispersion. The hydrophilic substance, which operates as the coating material, is selected from a wide variety of natural and synthetic polymers including shellacs, waxes, starches, cellulose acetates, phthalate or butyrate, polyvinyl-pyrrolidone, and polyvinyl chloride. After the coating material dissolves, the drug inside the microencapsule is immediately available for dissolution and absorption. Drug release, therefore, can be controlled by adjusting the thickness and dissolution rate of the coat. For example, the thickness can be varied from less than one μm to 200 μm by changing the amount of coating material from about 3 to 30 percent by weight of the total weight. By employing different thicknesses, typically three of four, the active agent will be released at different, predetermined times to afford a delayed release effect.

Approaches to further reducing the dissolution rate include, for example, coating the drug with a slowly dissolving material, or incorporating the drug into a formulation with a slowly dissolving carrier. Thus, encapsulated dissolution systems are prepared either by coating particles or granules of drug with varying thickness or slowly soluble polymers or by microencapsulation.

While it is contemplated that a unitary oral formulation containing both a leflunomide compound and a bioavailable pyrimidine compound provides ease of administration and patient compliance, it is also understood that the compounds may be administered separately but packaged together, e.g., in a blister pack, with instructions for administration.

Although examples of suitable dosage ranges are provided, it will be appreciated that the specific dosages administered in any given case will be adjusted in accordance with the specific compounds being administered, the disease to be treated, the condition of the subject and other relevant medical factors that may modify the activity of leflunomide, the response of the subject or the amount of bioavailable pyrimidine compound needed, as is well known by those skilled in the art. For example, the specific dose for a particular patient depends on age, body weight, general state of health, diet, the timing and mode of administration, the rate of excretion, and medicaments used in combination and the severity of the particular disorder to which the therapy is applied. Dosages for a given patient can be determined using conventional considerations, e.g., by customary comparison of the differential activities of the subject compounds and of a known agent, such as by means of an appropriate conventional pharmacological protocol.

The following examples are provide to assist in a further understanding of the invention. The particular materials and conditions employed are intended to be further illustrative of the invention and are not limiting upon the reasonable scope thereof.

Example 1: Effect of orotic acid administration on efficacy of leflunomide in the treatment of acute rejection

Lewis Rats which received heart transplants from Brown-Norway rats were observed for graft survival and inflammation (scored on a 0-3 scale, with 0 being no inflammation). Treatments included 0, 5, 10 or 15 mg/kg of leflunomide in combination with 0 or 100 mg/kg orotic acid. The results are tabulated below.

Number animals	Dose leflunomide (mg/kg/day)	Dose orotic acid (mg/kg/day)	Graft survival (days)	Inflammation score(Mean) (0-3)
8	0	0	6.9	3.0
8	5	0	>30	2.0
5	5	100	>30	2.3
5	10	0	>30	1.7
5	10	100	>30	1.8
5	15	0	>30	1.5
5	15	100	>30	1.6

Administration of leflunomide reduced the intensity of the rejection reaction, as shown by the inflammation score, in a dose-related fashion. Orotic acid did not significantly affect the efficacy of leflunomide to reduce the intensity of the rejection reaction.

Example 2: Effect of orotic acid on leflunomide toxicity as measured by changes in body weight

As noted previously, the most observed symptoms of experimental leflunomide-induced toxicity are anemia and diarrhea resulting in weight loss or reduced weight gain. Lewis rats with either an allograft or xenograft weighing between 200 and 235 grams were divided into four treatment groups. Each group received 30 mg/kg/day of leflunomide, a high, toxic dose: Group I received leflunomide only; Group II received leflunomide plus 36 mg/kg/day of sodium orotate by gavage; Group III received leflunomide plus 100 mg/kg/day of orotic acid by gavage; and Group IV received 250 mg/kg/day of uridine by IP injection. Weight of each rat was measured at week 1 and week 4 post-commencement of therapy. The results are tabulated below.

GROUP I (leflunomide only)

Treatment-dosage (mg/kg/day)	Weight at Week 1 Grams	Weight at Week 4 grams	Change in body weight
L30	234	246	13
L30	211	236	25
L30	241	237	-4
L 30	253	264	11
L 30	246	255	9
L 30	203	253	50
			Mean: 20.8

GROUP II

Treatment-dosage (mg/kg/day)	Weight at Week 1 Grams	Weight at Week 4 grams	Change in body weight
L30 + O36	270	266	-4
L30 + O36	244	235	-9
L30 + O36	250	218	-32
L30 + O36	205	267	62
L30 + O36	198	240	42
L30 + O36	211	262	51
			Mean: 18.3

GROUP III

Treatment-dosage (mg/kg/day)	Weight at Week 1 Grams	Weight at Week 4 grams	Change in body weight
L30 + O100	210	250	40
L30 + O100	208	265	57
L30 + O100	209	250	41
L30 + O100	207	271	66
L30 + O100	210	268	58
L30 + O100	197	258	61
			Mean: 53.7

GROUP IV

Treatment-dosage (mg/kg/day)	Weight at Week 1 Grams	Weight at Week 4 grams	Change in body weight
L30 + U250	207	231	24
L30 + U250	204	242	38
L30 + U250	208	230	22
L30 + U250	197	241	44
L30 + U250	204	272	68
L30 + U250	211	275	64
			Mean: 43.3

* L30 refers to administration of 30 mg/kg per day of leflunomide; O36 refers to 36 mg/kg per day of orotic acid; O100 refers to 100 mg/kg per day of orotic acid; and U250 refers to 250 mg/kg per day of uridine given IP.

The results showed that the use of a combination of leflunomide and orotic acid or a salt thereof significantly improved weight gain compared to use of leflunomide alone.

Example 3: Effect of orotic acid on leflunomide toxicity as measured by hematocrit

The experiment of Example 2 was repeated in Lewis rats and the hematocrit measured weekly for four weeks. The rats receiving treatment were divided into five groups wherein each group received 30/mg/kg/day leflunomide, a toxic, high dose. Group

I received leflunomide only. Group II received the leflunomide dose plus 36/mg/kg/day of sodium orotate; group III received the leflunomide dose plus 100mg/kg/day of sodium orotate; group IV received the leflunomide dose plus 88mg/kg/day of orotic acid; and group V received the leflunomide dose plus 250mg/kg/day of uridine given IP. A baseline hematocrit was measured, and hematocrits of each rat were measured at weeks 1-4 post commencement of therapy. The results are tabulated below.

GROUP I

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30	51	47	39	31	17
L30	57	52	46	33	20
L 30	54	55	54	37	30
L 30	55	49	48	33	24
L 30	52	50	46	37	22
L 30	53	53	44	41	30
					Mean: 23.8

GROUP II

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30 + O36	53	50	48	39	20
L30 + O36	57	50	49	32	14
L30 + O36	53	51	39	27	10
L30 + O36	54	52	49	47	44
L30 + O36	51	53	40	36	35
L30 + O36	52	49	44	30	20
					Mean: 23.8

GROUP III

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30 + O100	53	48	43	41	32
L30 + O100	52	49	51	46	44
L30 + O100	51	46	44	42	45
L30 + O100	57	53	50	41	33
L30 + O100	51	49	46	34	22
L30 + O100	52	51	53	43	46
					Mean: 37

GROUP IV

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30 + U250	52	47	48	50	46
L30 + U250	56	50	51	51	49
L30 + U250	54	46	43	41	25
L30 + U250	51	51	48	38	24
L30 + U250	53	49	54	51	44
L30 + U250	54	54	49	43	38
					Mean: 37.7

GROUP V

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30+ OA88	56	50	53	52	50
L30+ OA88	54	51	54	52	54
L30+ OA88	52	49	44	38	32
					Mean: 45.3

The results demonstrated that use of the combination of leflunomide and orotic acid or sodium orotate, provided significantly higher hematocrits than in the use of leflunomide alone.

As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to a composition containing "a pyrimidine compound" includes a mixture of two or more pyrimidine compounds. It should also be noted that the term "or" is generally employed in its sense including "and/or" unless the content clearly dictates otherwise.

All publications, patents and patent applications referenced in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications, patents and patent applications are herein expressly incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference. In case of conflict between the present disclosure and the incorporated patents, publications and references, the present disclosure should control.

The invention has been described with reference to various specific embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

5

We claim:

1. A pharmaceutical composition in unit dosage form for oral administration comprising an effective amount of a leflunomide compound; and an orally bioavailable pyrimidine compound, salts thereof or a combination thereof; together in a pharmaceutically acceptable carrier.

2. The composition of claim 1 wherein the pyrimidine compound is orotic acid, a salt thereof, triacetyluridine, a salt thereof, cytidine, a salt thereof, an acylated cytidine, a salt thereof, or a combination thereof.

3. The composition of claim 1, wherein the unit dosage contains 500 mg to 2000 mg of pyrimidine compound.

4. The composition of claim 1, wherein the leflunomide compound is leflunomide, A771726 or FK778.

5. The composition of claim 1, wherein the composition is formulated for controlled release.

6. The composition of claim 1, wherein the composition is formulated for rectal administration.

7. A pharmaceutical composition comprising a formulation for oral administration, the formulation comprising a therapeutically effective amount of leflunomide, and orotic acid or a salt thereof, and a pharmaceutically acceptable carrier.

8. A method of reducing toxicity associated with administration of a leflunomide compound to a patient in need thereof, comprising administering to the patient a toxicity-reducing amount of a bioavailable pyrimidine compound.

9. The method of claim 8, wherein the pyrimidine compound is orotic acid, a salt thereof, triacetyaluridine, a salt thereof, cytidine, a salt thereof, an acylated cytidine, a salt thereof, or a combination thereof.

10. The method of claim 8, wherein the pyrimidine compound is administered orally.

11. The method of claim 8, wherein the pyrimidine compound is administered in a daily dosage of from about 500 mg to about 2000 mg.

12. The method of claim 8, wherein the pyrimidine compound is co-administered substantially simultaneously with the leflunomide compound.

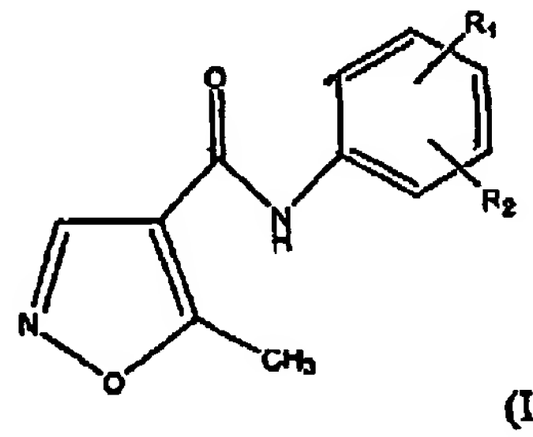
13. The method of claim 8, wherein the patient is a recipient of a transplant.

14. The method of claim 13, wherein the transplant is an allograft or a xenograft.

15. The method of claim 13, wherein the transplant is a heart, a kidney or bone marrow.

16. The method of claim 8, wherein the leflunomide compound is selected from a compound having

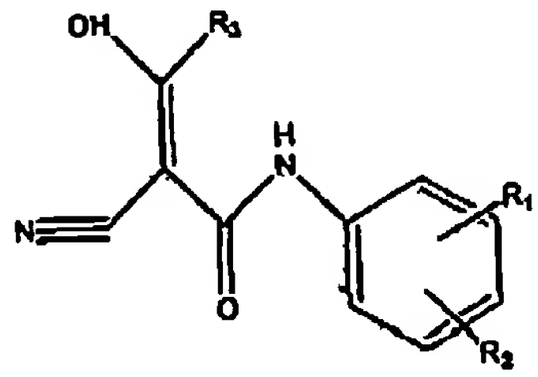
a) formula (II):



(II)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, $-NH-CO-CH_2Cl$ and $-NH-CO-CH_2Br$;

or formula (IV):

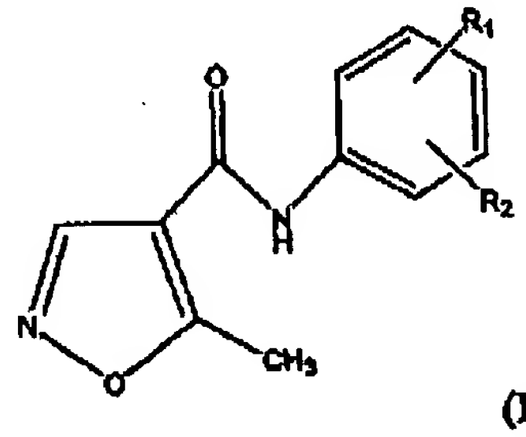


(IV)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, -NH-CO-CH_2Cl and -NH-CO-CH_2Br, and R3 is selected from the group consisting of C_{1-5} alkyl, C_{2-5} alkenyl, C_{2-5} alkynyl, and C_{3-6} cycloalkyl.

17. A method of extending the dosage range of a leflunomide compound comprising co-administering to a subject:

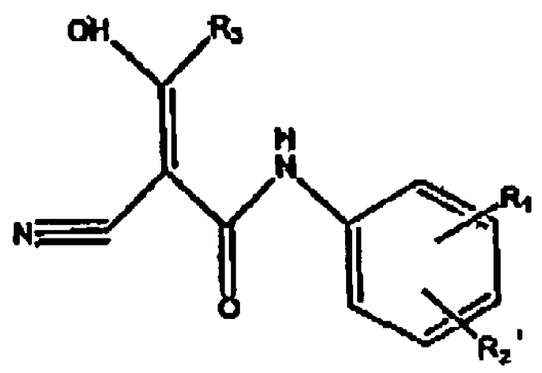
a) an effective amount of a leflunomide compound of formula (II):



(II)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, - -NH-CO-CH_2Cl and -NH-CO-CH_2Br;

or formula (IV):



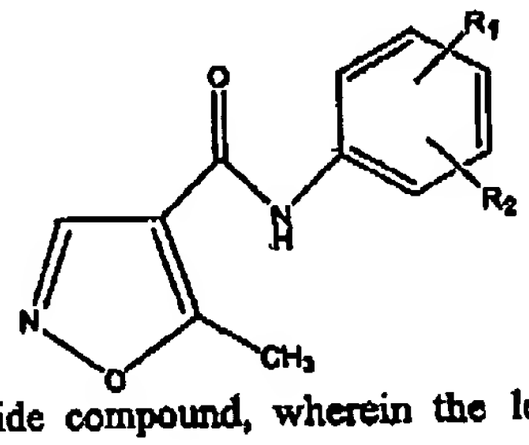
(IV)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, -NH-CO-CH_2Cl and -NH-CO-CH_2Br; and R_3 is selected from the group consisting of C_{1-5} alkyl, C_{2-5} alkenyl, C_{2-5} alkynyl, and C_{3-6} cycloalkyl; and

b) a toxicity-reducing amount of an orally bioavailable pyrimidine compound selected from the group consisting of orotic acid, a salt thereof, triacetyl uridine, a salt thereo, cytidine, a salt thereof, an acylated cytidine, a salt thereof, and a combination thereof.

18. A method of administering a toxic dose of a leflunomide compound to a mammal, comprising administering to the mammal an amount of an orally bioavailable pyrimidine compound sufficient to reduce the toxic effects of the leflunomide compound.

19. A method of reducing toxicity associated with the administration of a therapeutically effective amount of a leflunomide compound to a mammal, comprising: orally administering to the mammal a bioavailable pyrimidine compound selected from orotic acid, a salt thereof, triacetyluridine, , a salt thereof, cytidine, a salt thereof, an acylated cytidine, a salt thereof, and a combination thereof, in an amount effective to reduce the toxicity without blocking therapeutic

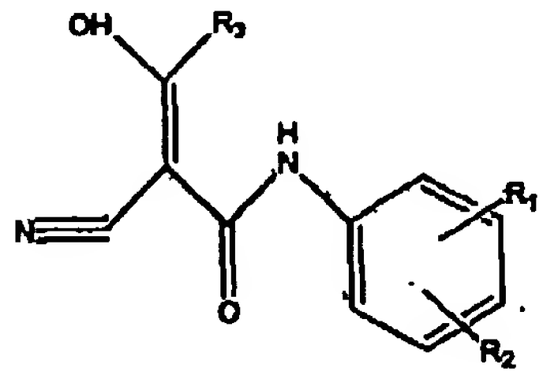


effect of the leflunomide compound, wherein the leflunomide compound is a compound of formula (II)

or formula (IV).

20. The method of claim 19 wherein the pyrimidine compound is orotic acid or a salt thereof.

21. A method of treating rejection in a transplant recipient comprising co-



administering a therapeutically effective amount of a leflunomide compound and a toxicity-reducing effective amount of bio-available pyrimidine compound.

22. The method of claim 21, wherein the pyrimidine compound is orally bio-available.

23. The method of claim 22, wherein the pyrimidine compound is orotic acid, a salt thereof, triacetyl uridine, a salt thereof, cytidine, a salt thereof, an acylated cytidine, a salt thereof, or a combination thereof.

24. A method of achieving an effect in a patient comprising co-administering an effective amount of a leflunomide compound and an effective amount of orotic acid, a salt thereof, triacetyl uridine, a salt thereof, or a combination thereof, wherein the effect is treatment of rejection of a transplant, wherein the transplant is heart, kidney or bone marrow.

25. A pharmaceutical combination comprising a packaging having a plurality containers, at least one container containing a leflunomide compound, at least one other container containing a bioavailable pyrimidine compound, and an instructions for co-administering the leflunomide compound and the pyrimidine compound to a subject who is a transplant recipient.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/26145

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 31/42
US CL : 514/378
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/378

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6,133,301 A (BARTLETT) 17 October 2000(17.10.2000), see entire document.	1-25

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search	Date of mailing of the international search report
26 November 2005 (26.11.2005)	08 DEC 2005
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer Michael Hartley Telephone No. (571) 272.1600

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